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123. THE EFFECT OF VARIATIONS IN FEEDING ON DAIRY COWS YIELDING MILK OF POOR QUALITY

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(With 4 Diagrams)

I. NATURE OF THE INVESTIGATION

THIS investigation was carried out with the object of finding a practicable means of improving the milk yield and the quality of the milk of poor milking cows. Four cows, two Friesians, cows A and B, one Guernsey, cow C, and a Shorthorn Cross, cow D, all of which were purchased from farmers in the Buchan district and which were giving milk consistently low in solids-not-fat, were used for the experiments. First of all the effect of good grazing with a supplementary ration was studied, then the effect of well-balanced normal rations and later experiments on the effect of high protein rations and high carbohydrate rations were carried out. The animals were under experiment during three lactations, 1931–3. The cows were milked twice daily, at 6.30 a.m. and 4.30 p.m., and the morning and evening milk samples were analysed daily for fat and solids-not-fat. The milk yield was also recorded.

II. PREVIOUS EXPERIENCE IN LOW BUTTERFAT AND SOLIDS-NOT-FAT PERCENTAGES

A large number of samples of milk from individual cows has been found to show either deficiencies in butterfat percentages, or solids-not-fat percentages, or both. These samples were taken at the byres by officials of local authorities, because of the frequency of cases of samples giving less than 3 per cent. fat and 8·5 per cent. solids-not-fat, which are the prescribed presumptive limits for unadulterated milk. The milk producer could not account for the poor quality of the milk by feeding, and often the sample was from a small herd. The byre samples analysed in these cases were found to be as poor and even poorer than the official samples suspected of being adulterated. It was thought, therefore, that an investigation on the changes, if any, in the yield and composition of such deficient milk due to feeding would be of practical value, particularly if cows whose milk was consistently below the presumptive limits were selected for the experiments.

Wing and Foord, of Cornell Agricultural Experiment Station(1), report that a herd of cows, which were poorly nourished, were fed liberally for 2 years with a resultant increase in the weekly average yield of milk of 46 lb., an increase of 2·4 lb. in the weekly average yield of fat. There is no record of an

increase in the solids-not-fat. Gowen and Tobey(2) found a distinct fall both in milk yield and lactose accompanied by an increase in total solids, fat and ash when cows were starved for 8 days with free access to water, while Cary and Meigs(3) showed that an increase in protein and energy contents of the ration produced a sharp rise in milk yield. Recently Nicholson and Lesser(4), using data from Friesians in Hampshire, found that addition of green foods, vitamins and minerals to the diet of cows which were giving milk deficient in solids-not-fat even under good management did not affect the solids-not-fat.

III. The effect of summer pasture feeding

At the commencement of this investigation the four cows A, B, C, D were put on an inadequate but not a starvation ration, consisting of 6 lb. sugar-beet pulp, 2 lb. bruised oats and 14 lb. straw, for a period of 28 days to determine whether this ration would have any serious effect on the milk yield and composition of the milk. The average values of the milk constituents before and during this period are given in Table I.

Table I

	Before reduced ration				During reduced ration (28 days)			
Cow	Fat %	S.N.F. %	Daily yield lb.	oz.	Fat %	S.N.F. %	Daily yield lb.	oz.
A, Friesian	3·09	7·85	16	12	3·23	7·99	15	8
B, ,,	2·90	7·75	16	10	2·97	7·69	15	4
C, Guernsey	3·50	7·79	10	0	3·42	8·05	10	8
D, Shorthorn Cross	3·49	7·96	14	8	3·27	7·85	15	10
Mean of 4 cows	3·25	7·84	14	8	3·22	7·90	14	6

These figures show that the reduction in the ration did not alter the milk yield or fat and solids-not-fat percentages. Before this experiment the cows had been fed on small farms in Buchan, and this reduced ration represented, more or less, their rations during the winter months, so that no abrupt changes in the milk yield or composition of the milk were expected.

In May 1931 the four cows were put out to pasture. Cows B and D, group 1, were on pasture only, day and night, for 7 weeks, while group 2, cows A and C and Carol, a Friesian, received a production ration in addition to the pasture for 7 weeks. At the end of the 7 weeks the groups were reversed. After the second 7 weeks' period the four cows A, B, C and D were kept on a well-balanced ration throughout the remainder of lactation 1931 and during the winter and spring months of lactation 1932, which commenced at the end of the year 1931. They were put out to pasture again in May 1932.

Results of pasture-feeding experiment

Table II gives the values for the milk constituents of individual cows and group averages during the reduced ration period and the following pasture period in 1931.

Both cows in group 1 and cow A in group 2 showed a marked rise in milk

yield when put out to pasture. The solids-not-fat percentage also rose with the milk yield, but the fat percentage remained unaltered. Cow C in group 2 showed no improvement in milk yield, but the milk yield in any case was very small. Cow C showed a rise in fat percentage, but there was no change in solids-not-fat percentage. Cow Carol was introduced here for comparative purposes, because she had been receiving a normal ration and was producing milk with a mean of 3·7 per cent. fat for the 2 months of March and April. Carol showed no change in solids-not-fat or milk yield during the pasture feeding, but the milk yield did not decline as would be expected with the advance of lactation.

Table II. *Lactation* 1931

	Reduced ration				Pasture			
	Fat %	S.N.F. %	Daily yield lb.	oz.	Fat %	S.N.F. %	Daily yield lb.	oz.
Group 1: Cow B	2·97	7·69	15	14	2·86	8·10	22	8
Cow D	3·27	7·85	15	10	3·22	8·05	20	8
Mean group 1	3·12	7·77	15	12	3·04	8·08	21	8
Group 1, pasture-reduced ration difference	−0·08	+0·31	+11	8	—	—	—	
Group 2: Cow A	3·23	7·99	15	8	3·44	8·49	22	2
Cow C	3·42	8·05	10	8	3·71	8·00	10	0
Carol	3·70	8·28	25	0	3·96	8·28	25	12
Mean group 2, omitting Carol	3·33	8·02	13	0	3·58	8·25	16	1
Group 2, pasture-reduced ration difference	+0·25	+0·23	+6	0	—	—	—	

The significance or non-significance of the difference between two mean values (fat percentage, solids-not-fat percentage, yield) was determined by Fisher's method (5), the probability of the significance or non-significance, *P*, being found from "Student's" table (6).

In group 1 the solids-not-fat rose by 0·31 during pasture feeding, while the total daily milk yield rose 11 lb. 8 oz., an increase of 30 per cent. The increase in solids-not-fat was found to be significant. In group 2 the increases of 0·25 and 0·23 in fat and solids-not-fat respectively given in Table II were also significant.

Comparing groups 1 and 2, both groups showed a rise in milk yield and solids-not-fat percentage when put out to pasture. Cows on pasture and a production ration showed also an increase in fat percentage. Most of the cows in both groups showed a sharp rise in milk yield and fat percentage within the first 6–9 days on grass, with a subsequent fall in fat percentage. When the groups were reversed, group 1 receiving the supplementary ration showed no difference in milk yield, fat or solids-not-fat percentages due to the addition or withdrawal of the supplementary ration.

Table III gives the mean values for the milk constituents of cows A, B, C and D during a month on winter rations and the following month on pasture during lactation 1932.

Table III. *Lactation* 1932

Cow	Winter rations (1 month) Fat %	S.N.F. %	Mean yield per milking lb.	Pasture (1 month) Fat %	S.N.F. %	Mean yield per milking lb.
A	3·77	8·42	11·66	3·83	8·55	13·04
B	2·90	8·18	9·30	3·02	8·14	10·32
C	3·86	8·16	5·16	4·26	8·60	4·92
D	3·04	8·39	19·63	3·26	8·38	20·67
Mean of 4 cows	3·42	8·29	11·44	3·59	8·47	12·24
Pasture-winter rations difference	+0·17	+0·18	+3·21	—	—	—

The increase in milk yield shown above on the change to pasture was not so marked as in 1931, and the mean rise in solids-not-fat for all cows was not significant, although there was a significant rise in solids-not-fat for cows A and C.

The general conclusions to be drawn from these observations seem to be that the change to pasture produces a decided improvement in the level of the solids-not-fat and the milk yield with previously poorly fed cows, but the improvement is not so marked with cows giving richer milk as shown with Carol in 1931, nor with cows which have been previously well fed as shown in lactation 1932 with the four cows. Chrzaszcz(7) found that an abrupt change from stall to pasture produced a fall in milk yield to 83–86 per cent. of the original yield and a rise in fat percentage. Johansson(8) also found that the fat content rose and the milk yield fell on the change to pasture, but further experiments of Johansson's(8) with two groups of six cows, one group of which had received no concentrates in their rations prior to the change to pasture and which had lost 2 kg. per head per day in milk yield, showed a rise in both fat content and milk yield on pasture. The results in this investigation show a similar rise in milk yield when dealing with poorly nourished cows, with also a rise in solids-not-fat. However, with none of the cows in both lactations was there found a drop in milk yield on change to pasture.

IV. Effect of well-balanced rations

A comparison was made between the corresponding mean values of fat, solids-not-fat and milk yield for the four cows A, B, C, D during lactation 1931 and lactation 1932 to determine the effect of feeding well-balanced rations during these 2 years. There is no record of the diet of the cows before their purchase during the first few months in lactation 1931. The comparison between the two lactations was made between the remaining months in lactation 1931 and the corresponding stage of the lactation in 1932.

The fortnightly means of fat and solids-not-fat percentages and the yield of the milk of the four cows during lactations 1931 and 1932 were calculated and plotted against the number of weeks in milk for both lactations, see

Diagrams I, II, III, IV. The significances of the differences between the means during the two lactations were calculated by Fisher's method. Their significances or non-significances are shown in Table IV.

Table IV. *Significances of the differences in fortnightly means of fat and solids-not-fat percentages and milk yield of cows A, B, C and D between lactations* 1931 *and* 1932

(Difference 1932–1931.)

Cow	Fat %	S.N.F. %	Yield per milking
A	Significant	Not significant	Probably significant
B	No difference	No difference	No difference
C	Probably significant	Significant	Significant
D	No difference	Not significant	Probably significant
Mean of 4 cows	Not significant	Significant	Significant

From this table it can be seen that there was a significant increase in solids-not-fat percentage and milk yield of the herd of four cows in 1932, but there was no improvement in the fat percentage. The milk of cow B alone showed no improvement. Diagrams I, II, III and IV show graphically what has been shown above by statistical analyses.

The rise in solids-not-fat percentage in 1932 might reasonably be attributed to the feeding of well-balanced rations, since Bartlett (9) has shown that the normal behaviour of fat and solids-not-fat percentage is to decline from the first lactation onwards. The increase in milk yield during 1932 may be due to the natural increase in yield which occurs in succeeding lactations until the cows have reached the limit of maximum production. This limit Bartlett (9) puts at the sixth lactation, while Tuff (10) puts it at the fifth. All of these four cows would possibly have reached their maximum in 1931, and if that is so, *the increase in yield is due to the better feeding during the latter part of lactation* 1931 *and the whole of lactation* 1932. Tocher (11) has shown that the correlation between milk yield and solids-not-fat is low, hence the rise in milk yield is not responsible for the rise in solids-not-fat. It should be noted that although the quality and quantity of the milk have improved the solids-not-fat percentage has not yet been raised to above the prescribed presumptive limit of 8·50.

V. Effect of high protein feeding

With the object of attempting to raise the solids-not-fat percentage to above the 8·50 limit the effect of a high protein ration was tried. Early work on the effect of protein in the diet of cows by Cary (12) and Cary and Meigs (3) indicated that a reduction of protein reduced the milk yield and milk nitrogen, while an increase in dietary protein had the opposite effect. The lactose content of the milk remained unchanged by alterations in the dietary protein. Olson (13) found that addition of protein in the form of soya beans produced an increase of 15 per cent. in fat percentage over a 10-day period. On the other hand Buschmann *et al.* (14) and Fries, Braman and Kriss (15) found that milk production was very little affected by changes in the amounts of protein supplied.

Recently Savage(16) reported on an experiment on feeding three groups of twelve cows each for a 3-year period on rations containing the same quantities of total digestible nutrients, but with varying amounts of total protein, viz. 16, 20 and 24 per cent. He found no significant differences in either milk or fat production between the three groups. This agrees with Henke and Goo's(17) observations that milk production, on rations containing 10·8 and 20 per cent. of digestible crude protein, was almost equal. However, Harrison, Savage

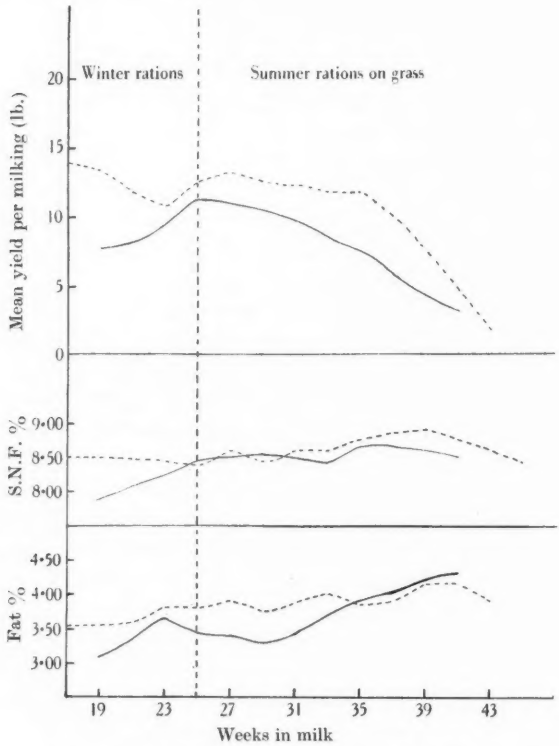

Diagram I. Cow A. Lactations 1931 and 1932. Fortnightly means.

—— Lactation 1931. - - - - Lactation 1932.

and Work(18) have shown recently that cows on 20 per cent. protein concentrates gave slightly higher milk yields than those on a 16 per cent. protein diet. None of these workers has been dealing with cows giving deficient milk, and it was thought possible that additional protein in the ration of the four cows in this experiment might have the effect of increasing the milk yield and milk nitrogen and hence the solids-not-fat percentage.

During the winter feeding in 1933 cows A, B and C, which were in milk, were given the following high protein ration (Table V) for a period of 40 days.

Table V. *High protein ration*

Feeding stuff	Albuminoids lb.	Oil lb.	Carbohydrates lb.	Fibre lb.
Decorticated earthnut cake, 8 lb.	3·20	0·66	0·60	0·06
Wheat bran, 3 lb.	0·28	0·08	1·19	0·05
Dried grains, 3 lb.	0·41	0·19	0·90	0·21
Turnips, 30 lb.	0·18	—	1·65	0·09
Hay, 12 lb.	0·65	0·12	3·08	1·80
Totals	4·72	1·05	7·42	2·21

Albuminoid ratio = 1 : 2·76.

This ration was constructed with the intention of giving the cows an adequate supply of all constituents with a 50 per cent. increase in the amount of protein to give a narrow albuminoid ratio. The cows were on a normal winter ration, both before and after this high protein ration. The milk was analysed daily for fat, solids-not-fat and twice weekly for lactose, casein, albumin and globulin and total nitrogen.

Results of high protein feeding

(1) *Effect on fat, solids-not-fat and yield of the milk.*

On examining the general trend of the milk yields for the three cows, no increase was apparent during the high protein feeding, and there was no indication that the gradual decrease in milk yield due to the advancing stage of lactation was retarded by the high protein ration. The fat percentage was unaffected, but there was a significant decrease in solids-not-fat percentage for the two cows A and B in the high protein ration period. Table VI gives, as a comparison between 40 days on a normal ration and the following 40 days on the high protein ration, the means of fat and solids-not-fat percentages and milk yield for cows A, B and C for the two periods.

Table VI

	Normal ration (40 days)			High protein ration (40 days)		
Cow	Fat %	S.N.F. %	Yield lb.	Fat %	S.N.F. %	Yield lb.
A	3·23	8·40	22·40	3·41	8·30	17·14
B	2·80	8·15	21·55	2·74	8·01	14·94
C	3·64	7·81	8·82	3·57	7·75	6·33
Mean of 3 cows	3·22	8·12	17·59	3·24	8·02	12·80

(2) *Effect on lactose, casein, albumin and globulin and total nitrogen contents of the milk.*

Table VII gives the mean values of lactose, casein, albumin and globulin and total nitrogen for the three cows during the normal ration and the following high protein ration and the significance of the differences in the means calculated from Fisher's P value.

It can be seen that there was a significant increase in total nitrogen and probably in albumin and globulin nitrogen, but no increase in the casein

Table VII

Cow	Lactose %		Casein N %		Albumin and globulin N %		Total N %	
	Normal ration	High protein	Normal ration	High protein	Normal ration	High protein	Normal ration	High protein
A	5·20	4·98	0·295	0·307	0·086	0·101	0·405	0·438
B	5·07	4·68	0·303	0·308	0·073	0·082	0·404	0·421
C	3·40	3·57	0·345	0·350	0·162	0·165	0·555	0·563
Mean of A, B, C	4·53	4·41	0·314	0·322	0·107	0·116	0·455	0·474
Difference H.P.–N.R.	Not significant		Not significant		Probably significant		Significant	

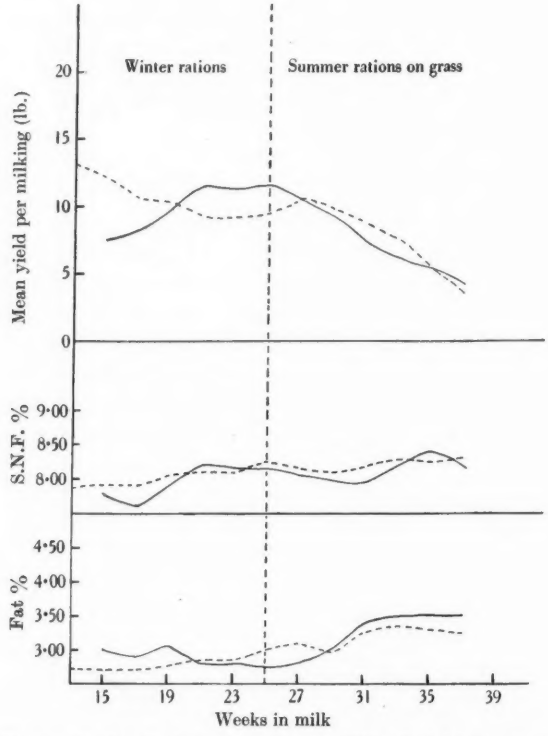

Diagram II. Cow B. Lactations 1931 and 1932. Fortnightly means.

—— Lactation 1931. - - - - Lactation 1932.

fraction during the high protein feeding. There was an almost significant decrease in the mean lactose for the three cows during the high protein ration, while for individual cows A and B there was a distinct fall in lactose. Excluding cow C, whose milk constituents were decidedly abnormal in their proportions, the decrease in the mean lactose percentage was significant. Tocher(11), in his statistical analyses of 676 samples of milk, showed that the lactose percentage decreased with a decrease in milk yield, and that the albumin and globulin

nitrogen and the total milk nitrogen gradually increased with a decrease in lactose. Here it was found that the total nitrogen and albumin and globulin nitrogen increased with a decrease in lactose. The decrease in lactose and the increases in total nitrogen and albumin and globulin nitrogen were greater than could be accounted for by the fall in milk yield, hence the changes must be due to the effect of feeding with a high protein ration.

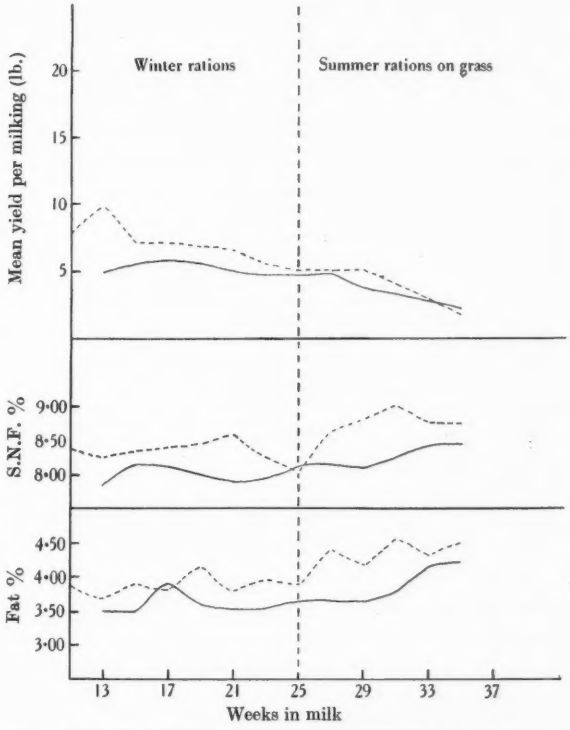

Diagram III. Cow C. Lactations 1931 and 1932. Fortnightly means.
—— Lactation 1931. - - - - Lactation 1932.

Taking into consideration all the results of feeding high protein rations to specially selected poor cows, the milk yield has not been significantly raised and the quality of the milk has been significantly lowered, as shown by the decrease in solids-not-fat, accompanied by a decrease in lactose and a rise in total milk nitrogen.

VI. Effect of high carbohydrate feeding

The effect on milk secretion of changing the dietary carbohydrate alone has been much less studied than the effects of changing the protein and fat. Small changes in the amount of carbohydrate have little effect either on the

composition or yield of the milk. Large reductions have been shown to cause a rapid fall in milk yield. Cary and Meigs(3) found that on reduction of the dietary carbohydrate the fat and lactose remained unaltered, while the milk nitrogen and milk yield were somewhat reduced.

Since the effect of a high protein ration with a narrow albuminoid ratio has been studied, it was thought that it would be of interest to study the effect of a high carbohydrate ration with a wide albuminoid ratio.

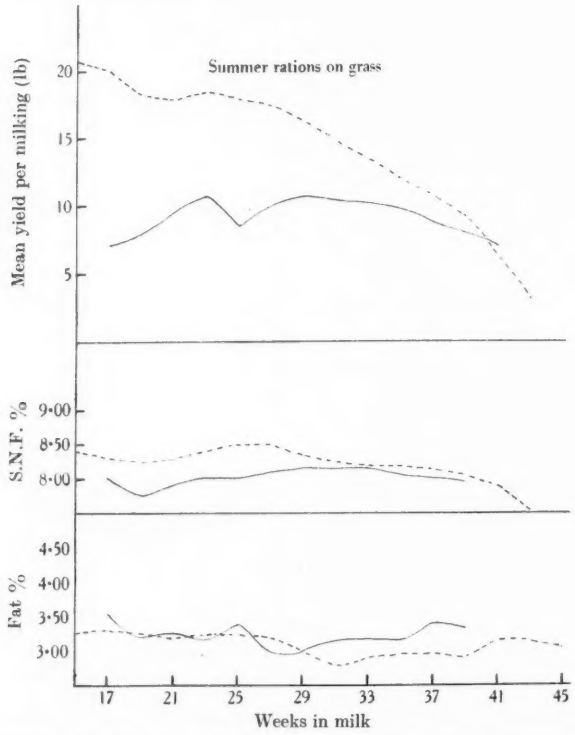

Diagram IV. Cow D. Lactations 1931 and 1932. Fortnightly means.

——— Lactation 1931. - - - - Lactation 1932.

This experiment was carried out in lactation 1933 during the summer feeding. The four cows A, B, C and D were on a normal pasture ration for 6 weeks after the high protein ration; they were then put on the following high carbohydrate ration for 9 weeks: *maintenance ration*, bare pasture; *production ration*, earthnut cake 1 part, bruised oats 3 parts, bran 2 parts, steamed bone flour 1 part, molassine meal 4 parts, the mixture being fed at the rate of $3\frac{1}{2}$ lb. per gallon. In addition each animal received 6 lb. treacle. On this basis of feeding the ration for a cow yielding 3 gallons contained 2·70 lb.

albuminoids, 0·77 lb. oil, 19·12 lb. carbohydrates and 5·85 lb. fibre, and had an albuminoid ratio of 1:9·90.

This ration gave normal quantities of oil and albuminoids and an increase in carbohydrate of 36 per cent. of normal, giving a wide albuminoid ratio.

Results of high carbohydrate ration

The effect of the high carbohydrate ration was studied by comparing the values for fat, solids-not-fat, and ash percentages and the milk yield found during the high carbohydrate feeding and during the two periods of normal rations, one preceding and one following the high carbohydrate ration. The mean values of fat, solids-not-fat, ash and milk yield for 4 weeks on the preceding normal ration and for the first 4 weeks on the high carbohydrate ration were calculated, also the means for the last 20 days on the high carbohydrate ration and the following 20 days on a normal ration, and are given in Table VIII.

Table VIII. *Means of fat percentage, solids-not-fat percentage, ash percentage, and yield of cows A, B and D during preceding and succeeding normal rations and the intervening high carbohydrate ration*

	Fat %	S.N.F. %	Ash %	Milk yield (lb.)
Preceding normal ration (4 weeks) (N.R.)	3·06	8·52	0·78	16·99
High carbohydrate ration (4 weeks) (H.C.)	3·24	8·24	0·73	14·26
Difference (H.C.–N.R.)	+0·18	−0·28	−0·05	−2·73
Result	Significant	Significant	—	—
Following normal ration (20 days) (N.R.)	3·55	8·27	0·76	10·87
High carbohydrate ration (20 days) (H.C.)	3·45	8·15	0·76	11·97
Difference (H.C.–N.R.)	−0·10	−0·12	−0·01	+1·10
Result	—	Significant	—	—

From this table, a significant increase in fat percentage is shown during the high carbohydrate ration in comparison with the fat percentage during the preceding normal ration. This difference was calculated, disregarding the effect of the fall in the milk yield on the fat percentage. Correcting the fat percentage for the fall in milk yield from Tocher's(11) regression curve gives only a mean difference of 0·06 per cent. fat between the two periods, which is not significant. There was no change in the fat when comparing the high carbohydrate period with the following normal period.

From the mean differences in solids-not-fat percentages between both the preceding and succeeding normal rations and the high carbohydrate period, *there is shown a significant decrease in solids-not-fat during the high carbohydrate feeding.* From Tocher's regression curve of solids-not-fat on milk yield, it is seen that there is a very slight increase in solids-not-fat as the milk yield decreases. In this experiment the solids-not-fat percentage decreased with decreasing yield, hence the decrease in solids-not-fat must be due to the ration. As regards the milk yield and ash percentage, the high carbohydrate ration had no effect.

The data for lactose, casein, albumin and globulin and total nitrogen during this experiment are scanty and no definite conclusions can be drawn, but on examining the general trend of the values there was an indication of an increase in lactose during the high carbohydrate period as shown by the means for cows A and B in Table IX.

Table IX. *Means of cows A and B*

	Lactose %	Casein N %	Albumin and globulin N %
Preceding normal ration (4 observations)	4·60	0·35	0·097
High carbohydrate ration (8 observations)	4·86	0·34	0·088
Succeeding normal ration (3 observations)	4·49	0·35	0·103

On consideration of all the experiments detailed here it seems that the milk of cows, which are giving milk deficient in solids-not-fat due partly to inadequate rations, can be improved in quality and quantity up to a certain limit by adequate feeding for a sufficient length of time. Experiments on feeding additional proteins and carbohydrates in the ration with a view to increasing still further the milk flow and the solids-not-fat have failed. The explanation of this inability to raise the solids-not-fat, even to the prescribed presumptive limit of 8·50 per cent., *is that heredity has a much greater influence on the milk-producing capacity of cows than environment. The solution to the problem of deficient milk must be the careful selection of cows with good milk records and a good milking pedigree.*

VII. Summary

Four cows, all giving milk low in solids-not-fat, were used for the experiment:

1. The change from winter rations to pasture produced a decided increase in the solids-not-fat percentage and milk yield.

2. Well-balanced rations fed to the cows during two lactations showed a small rise in the milk yield and solids-not-fat during the second lactation, but did not increase the solids-not-fat to above the 8·50 per cent. limit.

3. Feeding a high protein ration of albuminoid ratio 1:2·76 produced no change in milk yield or casein, decreased the solids-not-fat and lactose, and increased the total milk nitrogen and albumin and globulin nitrogen.

4. Feeding a high carbohydrate ration of albuminoid ratio 1:9·90 did not affect milk yield, fat or ash, but decreased slightly the solids-not-fat.

VIII. Acknowledgments

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124. THE METABOLISM OF BETAINE AND ALLIED TERTIARY NITROGENOUS BASES IN THE RUMINANT

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(With 1 Figure)

During the past few years considerable attention has been paid to the sporadic occurrence of a fishy flavour in the milk of cows fed on sugar-beet by-products, in which betaine in appreciable quantity is a common constituent. There is, on the average, about 1·5 per cent. of betaine nitrogen in beet molasses, 0·5 per cent. in molassed beet pulp and 0·03 per cent. in fresh beet tops (0·2 per cent. in the dry matter); dairy cows on normal rations containing suitable quantities of these products may be receiving up to 100 g. of betaine per day. The above feeding stuffs also contain traces of the tertiary nitrogenous bases, choline, trimethylamine and trimethylamine oxide.

The tertiary bases, especially betaine, are regarded as the precursors of the fishy taint of milk. In the investigation of the problem of fishy taint it was considered of fundamental importance to establish the fate of these bases in ruminant metabolism. The formation of a metabolite, more chemically active than betaine itself, was suspected, and it was considered that a clearer insight into the mechanism of the development of fishy flavour in milk could be obtained by studying the effect of small quantities of this metabolite entering the milk in any subsequent reactions which might occur in milk.

Very little attention has been paid to the quantitative aspects of the metabolism of tertiary bases in the higher animals, and the possibility of an innocuous tertiary base occurring in the urine as a common metabolite of all tertiary bases has not been explored. On the grounds of osmotic economy such a base would exist in urine in the molecular form and would only be slightly dissociated, if at all. The form of the free un-ionised tertiary base found in plants is betaine while, in the tissue and urine of some aquatic animals, a simpler form, trimethylamine oxide, exists. In the present work, the latter base has, so far as the ruminant is concerned, been found to be the main metabolite of all the tertiary bases tested.

Owing to the occurrence of betaine in the sugar beet, its utilisation by animals has been the subject of some investigation. Thus Velich and Stanek(1) found that the intravenous injection of betaine into the dog caused the whole of the betaine to be rapidly excreted in the urine, but that when fed orally only one-quarter was thus excreted. They also found that ruminants could cope more effectively with betaine and showed a marked degree of accommo-

dation to the feeding of regular amounts. A cow accustomed to beet molasses excreted no betaine, while a sheep excreted the base only during the first few days. Voltz (2) found that the whole of the betaine nitrogen was excreted by sheep even under conditions of low protein intake, only the non-nitrogenous portion of the molecule being utilised. Again Rieser (3) found that the creatine content of muscle rose on injecting betaine into rabbits, and he concluded that betaine condensed with a molecule of urea to form creatine.

Dorée and Galla (4) have found traces of trimethylamine in the blood and urine of higher animals, but Takeda (5) and Erdmann (6) could discover none in fresh material. Previous workers obtained considerable amounts of the base as a product of putrefaction or of destructive methods of distillation of these fluids. Suwa (7) isolated trimethylamine and unchanged trimethylamine oxide from the urine of rabbits injected with the oxide, while Kohlrausch (8) found that betaine fed to rabbits gave rise to trimethylamine. Langley (9) has suggested that bases such as betaine, choline, carnitine and trimethylaminocrotonic acid may decompose in a similar manner in the body to give rise to trimethylamine, and that the physiological functions of these substances may be associated with chemical changes involving the liberation of free trimethylamine. Langley fed trimethylamine to rabbits, and his maximum recovery of unchanged amine in the urine amounted to 19·3 per cent. He concluded that the bulk of the trimethylamine (80–96 per cent.) was wholly or partly demethylated, part being excreted in minute amount as secondary amine, none as primary amine, and the residue as urea. From his methods of analysis it can be gathered that the possible excretion of trimethylamine in the form of a detoxicated tertiary base, such as trimethylamine oxide, was not explored.

Trimethylamine oxide is of wide occurrence in the muscle and urine of some marine animals (Suwa (7), Grollmann (10)) and undoubtedly plays an important part in the excretion of tertiary nitrogen in fish generally. No reference has been made to its occurrence in the urine of higher animals, although Grollmann advances that possibility. As a free base, the oxide is feebly dissociated and it is doubtful if it is ionised at the *p*H of blood and urine. It can thus be considered an ideal form for the detoxication and excretion of highly ionised trimethylamine in the animal body, and it is possible that it enters into the physiological economy of tertiary nitrogen excretion in a similar way to urea in ammonia excretion. It seems surprising that this line of investigation has not been previously explored in studying the metabolism of tertiary bases. Further, the close similarity in properties of the oxide and of betaine may have led previous workers to mistake the excreted oxide in the urine for betaine itself.

It was originally intended in the present work to investigate the metabolism of betaine to the extent only of determining the amount of trimethylamine formed. In preliminary work, only small amounts of secondary and tertiary bases could be recovered from urine, but later, by reducing the urine from cows

(which had been given betaine) with zinc dust and alkali, zinc-copper couple or zinc and acetic acid, copious quantities of trimethylamine could be obtained. It was evident, therefore, that the main tertiary metabolite was a substance easily reduced to trimethylamine. This substance was found to be trimethylamine oxide, a fact which was confirmed in many instances by its isolation and by the analysis of its derivatives. The investigation was therefore widened to include the metabolism of choline, the methyl ester of dimethylaminoacetic acid (an isomer of betaine), trimethylamine oxide and trimethylamine.

The results justified this step, since they showed (*a*) that the main tertiary metabolite of betaine, choline and the ester was trimethylamine oxide, (*b*) that after feeding with trimethylamine oxide, it was quantitatively recovered unchanged in the urine, and (*c*) that when trimethylamine was fed, it was quantitatively recovered from the urine as trimethylamine oxide.

By taking periodic samples of urine after feeding, it was possible to find the time taken to reach the point of maximum excretion of the oxide, from which the rapidity of ingestion and metabolism of the base could be gauged. It was evident that a base requiring little or no metabolic change would be excreted rapidly. The rapidity with which the oxide was excreted after being fed afforded further evidence that this was the main tertiary metabolite of the more complex nitrogenous bases.

Experimental

(*a*) *Animals*

Shorthorn cows in full milk in the Institute herd were used as the experimental animals. The cows were in normal health, and gave normal milk as far as could be judged by chemical analysis.

(*b*) *The feeding of the nitrogenous bases*

All the experimental animals were on normal winter rations to which the bases were added as supplements. The requisite amount of the neutralised base was dissolved in water and used to moisten a small quantity of bran, which was then well mixed with 30 lb. of a mixture of silage, chaff and pulped roots. The solution of betaine, occurring commercially as the hydrochloride, was neutralised with a small excess of sodium bicarbonate solution. Trimethylamine oxide was fed as the free base and trimethylamine and choline as the neutral hydrochlorides. The ester was also neutralised with hydrochloric acid before incorporating into the ration. No difficulty was experienced in getting the animals to clear up their rations.

(The ester was prepared by the dry distillation of betaine at 300° C.; a 30 per cent. yield of a brown liquid contaminated with trimethylamine and having a fishy odour was obtained. By redistillation of the liquid (B.P. 135° C.) a clear liquid free from odour was obtained.)

(*c*) *Collection of urine*

Instead of the laborious procedure of waiting for the animals to pass urine naturally, it was fortunately found possible to make the animals pass their urine at reasonable intervals by gentle massage of the area immediately below the clitoris; in a short period almost all the animals experimented on grew tractable and responsive to this treatment. Urine was taken roughly every 30–45 min. and was bulked to form consecutive 90-min. samples. The periods of urine collection varied from 16 to 20 hours. Aliquot portions of each sample were analysed immediately and no preservative was used.

(*d*) *Methods of analysis*

The yield of urine at maximum output was roughly 1·2 litres per hour. Preliminary work showed that concentration of the acidified urine caused changes in the tertiary base distribution. Consequently aliquots of whole urine (200 ml.) were taken for analysis. The following analyses were carried out:

(i) *Free tertiary base nitrogen.* The samples were steam distilled in the presence of excess of magnesia, the volatile bases being collected in 0·1 *N* hydrochloric acid containing 20 ml. of 20 per cent. formalin previously neutralised to phenolphthalein.

(ii) *Tertiary base nitrogen from reducible base.* The residue from (i) was filtered by suction, the filtrate being acidified and concentrated to 150 ml. Excess of zinc powder and 2 g. of copper sulphate crystals were added and reduction allowed to occur for 16 hours. The volatile bases were steam distilled into 0·1 *N* acid containing neutral formalin. Before titrating the excess of standard acid in (i) and (ii), the distillates were boiled to drive off carbon dioxide, cooled, and 10 ml. of 20 per cent. formalin added before titration. This method of separation of ammonia and trimethylamine had been previously tested on mixtures of these volatile bases and found trustworthy.

(iii) *Residual "Stanek" nitrogen.* In some instances, the residue from (ii) was filtered, concentrated and treated with Stanek's reagent (a concentrated iodine-potassium iodide solution) and the nitrogen thus precipitated determined by the Kjeldahl method.

In some instances the presence of secondary amines in the distillate of (i) was also investigated.

(iv) The *creatinine content* was determined colorimetrically by Folin's method. No significance was ascribed to this value other than evidence of the occurrence of diuresis due to the nature of the material fed. The neutralisation of betaine hydrochloride before feeding meant the inclusion of a large amount of common salt in the ration which would not occur when the betaine-containing food was fed. The salt caused considerable diuresis, with the result that the intensity of the excretion of trimethylamine oxide at the peak period was decreased. In general practice, however, it has generally been observed

that animals fed on beet by-products give a more dilute urine than those fed on the usual feeding stuffs.

(e) *Quantities of the materials fed*

Beet molasses contains from 6 to 7 per cent. of betaine, while dried molassed beet pulp contains 30 per cent. of the original molasses and therefore from 2·0 to 2·4 per cent. of betaine. At normal rates of the feeding of these feeding stuffs the daily betaine intake is roughly 100 g. Betaine has no physiological action and can be fed in larger quantities, and therefore in the two experiments carried out with betaine, 156 g. (18·7 g. N) and 230 g. (27·5 g. N) were fed as one dose. With trimethylamine oxide, which also possesses no physiological action, 25 g. (4·7 g. N) were fed. With choline, 25 g. of the hydrochloride (2·22 g. N) were fed, while with trimethylamine, 30 g. of the hydrochloride (4·4 g. N) were given. It is interesting to note that the maximum pharmacological dose of trimethylamine for man is 0·5 g. of the hydrochloride. On a generous estimate of a cow's weight being ten times that of man, it was found that six times the pharmacological dose for a cow was given without signs of discomfort; the milk yield, which rapidly diminishes under abnormal conditions, was not affected. In the two experiments in which the methyl ester of dimethyl aminoacetic acid was fed, 12 and 7·28 g. of the ester (1·44 and 0·85 g. of tertiary nitrogen respectively) were given in the feed.

Experimental results

Tables I and II give the results of the experiments on feeding betaine (156 and 230 g. of betaine respectively).

Table I. *First betaine-feeding experiment*

156 g. betaine (=18·7 g. N). Cow: Lena 3, wt. 1264 lb.

Urine sample No.	Time after feeding hours	Volume of urine ml.	Free $N(CH_3)_3$ nitrogen ml.	Free $N(CH_3)_3$ nitrogen per 100 ml. mg.	$N(CH_3)_3O$ nitrogen mg.	$N(CH_3)_3O$ nitrogen per 100 ml. mg.	Residual "Stanek" nitrogen mg.
0 (control)	—	—	—	1·7	Nil	Nil	—
1	1·5	1170	42	3·6	41	3·5	550
2	2·6	1340	31	2·3	116	8·7	619
3	3·6	860	26	3·0	214	25·5	119
4	4·9	400	12	3·0	194	57·1	476
5	6·1	1500	42	2·8	684	45·6	338
6	7·4	1340	39	2·9	437	32·6	434
7	8·8	1000	30	3·0	596	60·8	372
8	10·0	710	20	2·8	553	77·9	158
9 (control)	36	—	—	1·9	—	4·1	—
Total			242		2835		

Total tertiary nitrogen recovered = 3·08 g. (=16·5 per cent.).
Ratio $N(CH_3)_3O$ nitrogen to $N(CH_3)_3$ nitrogen = 12 : 1.

Table III gives the results of feeding 30 g. of trimethylamine hydrochloride (=4·42 g. of nitrogen); Table IV gives the results of feeding 25 g. of trimethylamine oxide (=4·67 g. of nitrogen). The feeding of these two compounds

gave 93 and 95·6 per cent. recovery of tertiary nitrogen respectively in 16-hour periods of urine collection. The peak of excretion occurred 6·2 and 4·5 hours respectively after feeding.

Table II. *Second betaine-feeding experiment*

Amount fed = 230 g. betaine (= 27·5 g. N). Cow: Flora 6, wt. 1356 lb.

Urine sample No.	Time after feeding hours	Volume of urine litres	Free $N(CH_3)_3$ nitrogen mg.	Free $N(CH_3)_3$ nitrogen per 100 ml. urine mg.	$N(CH_3)_3O$ nitrogen mg.	$N(CH_3)_3O$ nitrogen per 100 ml. mg.	Residual "Stanek" nitrogen mg.	Creatinine nitrogen per 100 ml. mg.
0 (control)	—	—	—	1·0	—	—	—	172
1	1·6	1·04	1·1	0·1	10	—	530	171
2	2·7	0·83	2·6	0·3	27	—	294	169
3	3·8	0·37	3·7	1·0	235	—	89	46
4	5·1	0·37	2·9	0·8			124	41
5	6·8	0·62	5·0	0·8	337	—	375	40
6	8·3	0·12	1·5	1·3	372	—	54	42
7	9·8	0·48	14·9	3·1			198	45 *
8	12·0	1·34	28·4	2·1	1185	—	838	73
9	14·1	1·35	28·4	2·1	729	—	1512	78
10	16·2	1·32	43·5	3·3	641	—	779	81
11	19·0	1·02	29·5	2·9	224	—	512	75
12 (control)	40	—	—	0·9	—	3·7	—	181

* Considerable diuresis.

Total tertiary nitrogen recovered = 3·922 g. (= 14·3 per cent.).
$N(CH_3)_3O$ nitrogen : $N(CH_3)_3$ nitrogen = 23 : 1.

Table III. *Feeding of trimethylamine hydrochloride*

Amount fed = 30 g. $N(CH_3)_3HCl$ (= 4·42 g. N). Period of urine collection = 16 hours. Cow: Lily 4.

Urine sample No.	Time after feeding hours	Volume of urine litres	Total free $N(CH_3)_3$ nitrogen mg.	Free $N(CH_3)_3$ nitrogen per 100 ml. urine mg.	Total $N(CH_3)_3O$ nitrogen mg.	$N(CH_3)_3O$ nitrogen per 100 ml. urine mg.	Creatinine per 100 ml. urine mg.
0 (control)	—	—	—	0·7	—	—	120
1	1·3	0·70	6·2	0·9	34	5·0	96
2	2·3	0·80	5·1	0·6	24	3·0	83
3	3·3	0·80	4·2	0·5	206	25·7	99
4	4·5	0·70	12·8	1·8	359	51·3	78
5	6·2	0·90	18·8	2·1	607	67·5	148
6	7·5	1·20	27·5	2·3	518	43·2	87
7	8·9	1·60	65·3	3·1	692	43·2	69
8	11·3	1·60	59·7	3·7	684	42·8	125
9	13·5	1·25	32·8	2·6	457	36·5	140
10	16·0	1·35	34·0	2·5	253	18·8	118
11 (control)	30	—	—	0·4	—	5·0	176
Total		10·90	266		3834		

Tertiary nitrogen recovered in 16 hours = 4·10 g. (93 per cent.).
Ratio of $N(CH_3)_3O$ to $N(CH_3)_3$ nitrogen = 14 : 1.
Peak of excretion: 6 hours after feeding.

Table V gives the results of feeding choline hydrochloride and Table VI of feeding the methyl ester of dimethyl aminoacetic acid.

Table IV. *Feeding of trimethylamine oxide*

Amount fed = 25 g. $N(CH_3)_3O$ (= 4·67 g. N). Cow: Flora 6.

Urine sample No.	Time after feeding hours	Volume of urine litres	Total free $N(CH_3)_3$ nitrogen mg.	$N(CH_3)_3$ nitrogen per 100 ml. urine mg.	Total $N(CH_3)_3O$ nitrogen mg.	$N(CH_3)_3O$ nitrogen per 100 ml. urine mg.	Creatinine per 100 ml. mg.
0 (control)	—	—	—	0·7	—	4	125
1	1·1	0·70	5·8	0·8	69	10	130
2	2·3	0·65	8·7	1·3	90	14	69
3	3·3	1·55	17·4	1·1	391	27	37
4	4·5	0·72	21·5	3·0	980	136	92
5	5·7	1·10	30·9	2·8	596	54	55
6	7·0	1·00	38·8	3·9	561	56	68
7	8·3	0·75	23·4	3·1	433	58	90
8	9·6	0·80	43·9	3·8	520	65	100
9	11·6	1·05	45·4	4·3	177	17	91
10	14·5	0·94	37·0	4·0	299	32	86
11	16·0	0·60	17·2	2·9	63	11	74
12 (control)	30	—	—	0·6	—	3	110

Tertiary nitrogen recovered = 4·47 g. (95·6 per cent.).
Ratio of $N(CH_3)_3O$ nitrogen to $N(CH_3)_3$ nitrogen = 15 : 1.
Peak of excretion: 4·5 hours after feeding.

Table V. *Feeding of choline hydrochloride*

Amount fed = 25 g. (= 2·22 g. N). Cow: Phosphor.

Urine sample No.	Time after feeding hours	Volume of urine litres	Free $N(CH_3)_3$ nitrogen mg.	Free $N(CH_3)_3$ nitrogen per 100 ml. mg.	$N(CH_3)_3O$ nitrogen mg.	$N(CH_3)_3O$ nitrogen per 100 ml. mg.
0 (control)	—	—	—	—	—	—
1	1·0	0·33	3·4	1·03	31·5	9·7
2	2·0	0·40	7·0	1·74	82·0	20·5
3	3·3	0·66	6·3	0·96	163·7	24·8
4	4·6	0·40	6·4	1·59	203·2	50·8
5	6·5	0·40	14·2	3·55	126·0	31·5
6	7·7	0·33	9·0	2·72	98·3	29·8
7	9·1	0·55	12·4	2·25	84·7	15·4
8	10·8	0·25	10·4	4·17	82·0	32·8
9	11·9	0·33	9·4	2·90	62·0	38·0
Total		3·65	79·0		933·0	

Tertiary nitrogen recovered = 1·012 g. = 45·6 per cent. in 12 hours.
$N(CH_3)_3O$ nitrogen: $N(CH_3)_3$ nitrogen = 11·8 : 1.
Peak of excretion of $N(CH_3)_3O$ = 4·6 hours after feeding.

The rates of excretion of trimethylamine oxide in the case of all the bases investigated are plotted in Fig. 1.

The occurrence of other methylated nitrogenous bases in the urine

Some of the samples of urine from the betaine-feeding experiments were investigated for the presence of mono- and dimethylamine. The solutions of mixed volatile bases distilled from various urine samples were treated with sodium nitrite and acetic acid, and the trimethylamine and any nitrosamine steam-distilled after rendering alkaline. The difference between the trimethylamine value and the trimethylamine *plus* dimethylamine values (after

Table VI. *Feeding of the methyl ester of dimethylaminoacetic acid*

Urine sample No.	Time after feeding hours	Volume of urine litres	Free $N(CH_3)_3$ nitrogen mg.	Free $N(CH_3)_3$ nitrogen per 100 ml. urine mg.	$N(CH_3)_3O$ nitrogen mg.	$N(CH_3)_3O$ nitrogen per 100 ml. urine mg.
	I. 7·28 g. of ester (=0·85 g. N). Cow: Lottie 8.					
0 (control)	—	—	—	0·18	—	1·4
1	3·3	1·09	3·4	0·31	12·2	3·9
2	5·3	0·82	3·4	0·42	67·7	8·3
3	7·0	1·09	2·8	0·26	57·6	5·3
4	8·9	1·08	1·6	0·15	38·1	3·6
5	10·3	0·90	5·3	0·59	33·5	3·7
6	12·8	1·15	6·2	0·54	40·6	3·5
7 (control)	40·0	—	—	0·21	—	0·2
	II. 12·0 g. of ester (=1·44 g. N). Cow: Lottie 8.					
0 (control)	—	—	—	0·08	—	0·9
1	3·0	0·90	2·0	0·23	25·6	2·8
2	5·8	1·54	12·1	0·78	59·4	3·9
3	7·8	1·15	5·0	0·43	26·7	2·3
4	9·5	0·55	4·0	0·72	27·1	4·9
5	12·2	1·02	5·2	0·51	56·6	5·5
6 (control)	40·0	—	—	0·20	—	0·4

Tertiary nitrogen recovered: I, 0·303 g. (=3·57 per cent.); II, 0·233 g. (=16·2 per cent.).

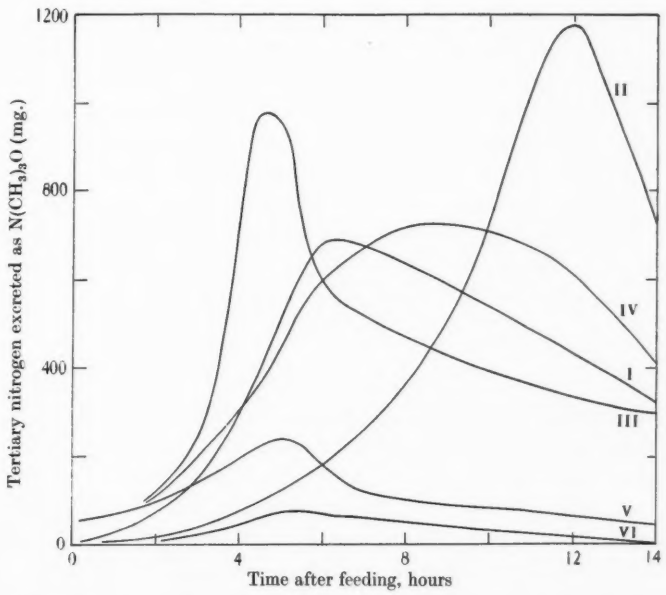

Fig. 1. Rates of excretion of trimethylamine oxide nitrogen on feeding various tertiary nitrogenous bases. I, betaine (18·7 g. N); II, betaine (27·5 g. N); III, trimethylamine oxide (4·67 g. N); IV, trimethylamine hydrochloride (4·42 g. N); V, choline hydrochloride (2·22 g. N); V, methyl dimethylaminoacetate (0·85 g. N).

reduction of the nitrosamine with zinc and acetic acid) gave the amount of dimethylamine present, while the total volatile base after absorption of the ammonia by freshly precipitated mercuric oxide gave, after subtraction of the values of the other methylated nitrogen bases, the monomethylamine value.

The following amounts of CH_3NH_2 and $(CH_3)_2NH$ per 100 ml. of urine were found present:

Control urine samples:

CH_3NH_2 nitrogen: 0·07, 0·09, 0·09 mg. N;

$(CH_3)_2NH$ nitrogen: 0·09, 0·08, 0·09 mg. N.

Urine samples at peak excretion of $N(CH_3)_3O$ nitrogen:

CH_3NH_2 nitrogen: 0·11, 0·12, 0·09, 0·08 mg. N;

$(CH_3)_2NH$ nitrogen: 0·13, 0·14, 0·10, 0·14 mg. N.

For both forms of nitrogen, the amounts in urine were of a low order, especially when compared with the amounts of trimethylamine oxide present. There were slightly larger amounts in the experimental urine samples than in the control samples; this extra amount, however, accounted only for an insignificant amount of the total tertiary nitrogen fed to the animal.

Characterisation of the trimethylamine oxide in the urine

Trimethylamine oxide was isolated as picrate from samples of urine taken at the peak excretion of the base. The base was precipitated with phosphotungstic acid and, after liberation with baryta, treated with Neuberg's reagent to separate it from other bases. The base was then precipitated as its picrate (fine needles, M.P. 197° C.). Ether extraction of the picric acid from acid solution yielded a solution which, on reduction with zinc-copper couple, gave all its nitrogen as the volatile base, trimethylamine. The aurichloride, prepared in two cases, gave a melting point of 249–251° C.

Owing to the difficulties of separating the relatively small quantities of trimethylamine oxide from urine, the yields of picrate were not quantitative, but the above isolations of crystalline derivatives, together with the formation of trimethylamine on reduction with zinc-copper couple, were considered sufficient evidence of the metabolite being trimethylamine oxide.

Discussion of results

The rapid and almost quantitative excretion of trimethylamine oxide in the unchanged form and also the rapid excretion of trimethylamine as its oxide prove that this is the mode of excretion of tertiary nitrogenous bases in the ruminant, and that with these simple bases no attack on the methyl groups attached to the nitrogen occurs. But when one of the groups attached to the nitrogen is a substituted methyl group (in which one H atom is substituted by a CH_2OH or COOH group), there is a varying and considerable metabolism of both methyl and substituted methyl groups; from 14 to 33 per cent. only of the nitrogen appears in the urine in the tertiary form, while the amounts of other forms of methylated nitrogen are insignificant. The metabolic

nitrogen must therefore appear in the urine in molecular form, *i.e.* either as trimethylamine oxide or as urea. The greater utilisation of betaine by animals fed regularly on a betaine-containing food is explained by their greater capacity to metabolise the nitrogen to urea and not to trimethylamine oxide, but this will naturally depend on the level of intake of betaine nitrogen.

The trimethylamine obtained by distillation of the urine samples with magnesia varied generally with the content of trimethylamine oxide in the urine. It is possible that all the trimethylamine determined by this method did not exist as such in the urine, but that a small amount of reduction of the oxide by the reducing agents in cow's urine (sugars, etc., in alkaline solution) contributed to the value. This matter has not been further investigated. In most cases the ratio of $N(CH_3)O$ nitrogen to $N(CH_3)_3$ nitrogen was from 12 to 15 to 1. Large amounts of trimethylamine were formed by bacterial action on the stored urine samples.

The occurrence of unchanged betaine in the urine was extensively investigated. In Tables I and II are given values for residual "Stanek" nitrogen. Betaine gives completely crystalline precipitates with this reagent, but the material separating from urine (after elimination of trimethylamine oxide and concentration) was without exception a dark brownish red viscous oil which was easily separated by filtering, but which could not be induced to crystallise either by adding iodine powder or some betaine iodide crystals. Further, on freeing the oil from iodine, the nitrogenous compound thus isolated was almost completely precipitated by Neuberg's reagent. This proved that the betaine content of the oil, if any, was very low. From a number of observations the conclusion was reached that there could be very little unchanged betaine in the experimental urine. It can also be seen from Tables I and II that the control urine samples contained residual "Stanek" nitrogen in amounts which were of the same order as those for the experimental urine.

The rates of excretion of trimethylamine oxide show well-defined peaks at various times after feeding, depending on the amount and form of tertiary nitrogen fed to the animal. With the simpler bases, $N(CH_3)_3$ and $N(CH_3)_3O$, the maximum rates of excretion of the oxide occur at about $4\frac{1}{2}$–6 hours after feeding. When feeding small amounts of tertiary nitrogen as choline or the methyl ester of dimethylaminoacetic acid, the peak again occurred about $4\frac{1}{2}$–6 hours after feeding. But when comparatively large amounts of betaine were fed, the peak of excretion was pushed forward to about 12 hours after feeding (Table II); the feeding of lower amounts of betaine caused the peak to be reached in a shorter time after feeding (6·1 hours, Table I). For an intake of 100 g. of betaine daily, it can be inferred that the peak of excretion would be reached in 4–$4\frac{1}{2}$ hours.

The importance of a peak of excretion of the oxide rests in the fact that the maximum concentration of the metabolite would occur in the blood of the animal shortly before the peak of excretion in the urine would be reached. The maximum concentration of the oxide in the "residual nitrogen" fraction

of milk secreted would therefore be expected to occur at this time. Since the trimethylamine oxide in milk is in all probability the precursor of the fishy taint, it is evident that the possibilities of the development of the taint are greatest when the cow is milked just previous to the peak of oxide excretion in the urine. The importance of the time interval between feeding the betaine-containing food and milking is obvious, and with this is also associated the amount of betaine fed. With an intake of 100 g. of betaine the peak is reached in roughly $4\frac{1}{2}$ hours, and it is therefore advisable to feed beet by-products as far away from the subsequent milking time as possible, namely, during or soon after the milking time.

The mechanism of the development of the fishy taint in milk by interaction of the precursor with one or more of the milk constituents is being at present investigated. The results of this work will be reported in a later paper.

SUMMARY

In the ruminant, the main tertiary nitrogenous metabolite of all the tertiary nitrogenous bases examined is trimethylamine oxide. Small traces of trimethylamine and insignificant traces of mono- and dimethylamine also occur in cow's urine. The nitrogen of simple bases (trimethylamine and trimethylamine oxide) is almost quantitatively and rapidly excreted as trimethylamine oxide. With betaine, choline and the methyl ester of dimethylaminoacetic acid, only from 14 to 43 per cent. of the nitrogen is excreted as trimethylamine oxide, the amount so excreted depending on the level of nitrogen intake, the nature of the base fed and the degree of accommodation of the animal to the base fed.

The rates of excretion of the tertiary metabolite show well-defined peaks depending on the nature and amount of the base fed. The feeding of 100 g. of betaine would show a peak of excretion at about $4\frac{1}{2}$ hours after feeding. The importance of the time interval between feeding and milking is discussed from this aspect. No unchanged betaine was be detected in the urine.

The author is indebted to the Sugar Beet Research and Education Committee (Ministry of Agriculture and Fisheries) for a grant to carry out this work.

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637.127.6

125. TECHNIQUE FOR OBTAINING ANAEROBIC MILK, WITH SOME OBSERVATIONS ON ITS CO_2 CONTENT

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(With 2 Figures)

The early attempts of Hoppe, Stechnow and Pflüger to draw milk anaerobically are critically reviewed by Marshall(1), whose apparatus appears satisfactory but unnecessarily complicated. Van Slyke and Baker(2) describe a method they used in their estimation of carbonic acid and carbonates in milk, but since the milk fills their containing vessel by displacement of air it must of necessity come in contact with air, and therefore their technique was considered inadequate.

A simple method has been devised, an adaptation of the apparatus used by Austin *et al.*(3) for the collection of blood.

A heavy glass tube $\frac{3}{4}$ in. in diameter and 6 in. in length (see Fig. 1) was fitted at the bottom with a stopcock and at the top with a three-way capillary stopcock to which was attached about a foot of rubber pressure tubing at the end of which was a milk tube. The bottom stopcock was attached to a mercury leveller. By manipulation of the mercury, air is driven out of the system and the collecting tube filled with mercury up to the three-way stopcock. Several streams of milk are then expelled from the udder by hand to ensure securing a normal sample. The milk tube, previously sterilised in alcohol, is then carefully inserted in the teat duct and held in place. The three-way stopcock is so adjusted that the first milk escapes through the vent, there being sufficient force within the udder to expel it. About 20 ml. of milk is discarded in this manner, sufficient to ensure that all air is flushed out of the tubing above the stopcock. The mercury leveller is then lowered and the stopcock adjusted so that the milk flows into the collecting tube as the mercury runs out. The advantages of this method are threefold, for milk may thus be drawn anaerobically into an oxidation-reduction indicator, transferred into an electrode vessel, or into a van Slyke apparatus for gas analysis.

Drawing milk anaerobically into oxidation-reduction indicators

The indicator is placed in the bulb of the collecting tube which is de-aerated by shaking under Torricellian vacuum. Milk is then drawn anaerobically in the manner described.

Transferring anaerobically drawn milk to an electrode vessel

The electrode vessel consists of a glass tube with a stopcock at either end. In the tube is fused a platinum electrode and a glass tube to hold potassium chloride agar. Milk is drawn anaerobically in the manner described. The milk

tube is then removed and the pressure tubing attached to stopcock *A* of the electrode vessel (see Fig. 2). Nitrogen, which has been passed over hot copper, is then introduced through the side arm of the three-way stopcock and allowed to flush the electrode vessel. The three-way stopcock is then readjusted and the milk forced up into the electrode vessel under mercury.

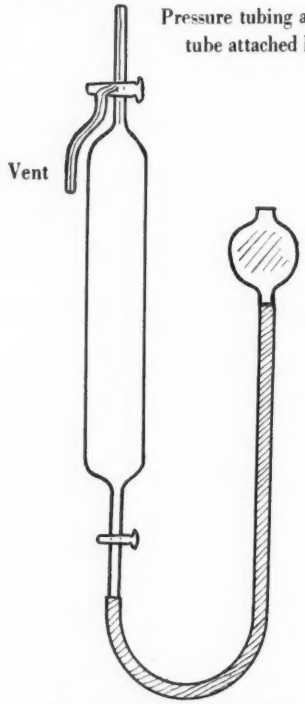

Fig. 1. Apparatus for the anaerobic withdrawal of milk.

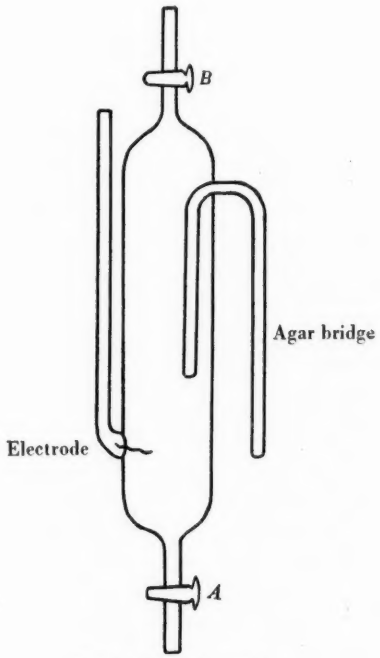

Fig. 2. Electrode vessel.

Transferring anaerobically drawn milk into the van Slyke apparatus

The milk tube is removed and the pressure tubing attached to the side arm of the van Slyke apparatus. Mercury is drawn up through the side arm of the collecting tube till the connection between the two pieces of apparatus is full of mercury. The milk may then be run into the van Slyke apparatus under mercury by adjusting the three-way stopcock of the collecting tube and raising the mercury leveller, while lowering that of the van Slyke apparatus.

Total CO_2 determinations

Gas determinations on six anaerobically drawn milks were done in a manner similar to that for the estimation of CO_2 in blood plasma, with the exception that 20 per cent. lactic acid was used in place of 5 per cent. sulphuric

acid. Table I gives the total CO_2 of the blood plasma of six cows and the corresponding CO_2 determinations of their milks drawn anaerobically at the same time.

Table I

Sample	Blood plasma CO_2 vol. %	Milk CO_2 vol. %
1	54·1	25·8
2	53·8	26·8
3	59·8	28·7
4	57·9	25·8
5	59·8	25·8
6	58·9	27·7

Discussion

Van Slyke and Baker (2) report that the amount of carbon dioxide present in normal milk in the udder varied between 8 and 85 vol. per cent. This variation seems surprisingly great, for the "fixed" bases with which the CO_2 is mainly combined remain relatively constant. Table II, calculated from data given in *Fundamentals of Dairy Science* (4), shows approximately the fixed bases present in milk and in blood.

Table II

	Milk serum (van Slyke and Bosworth (5)) g. per litre	Blood serum (Abderhalden (6)) g. per litre
Na	0·57	3·20
K	1·24	0·21
Ca	0·46	0·08

The Ca in milk, which appears to be mostly held by the prosthetic group of caseinogen, can perhaps be largely disregarded as a binder of CO_2 in milk. Confining our attention then solely to Na and K we see that there is just approximately half the concentration of these elements in milk that there is in blood serum. This suggests that the CO_2 content of blood should be roughly twice that of milk. The estimations reported here show this to be the case.

Summary

1. A method has been described for obtaining milk anaerobically from the udder.

2. A technique for transferring anaerobically drawn milk to an electrode vessel for the determination of oxidation-reduction potentials under anaerobic conditions has been described.

3. A technique for transferring anaerobically drawn milk to a van Slyke apparatus for gas analysis has been described.

4. The total CO_2 of cows' milk is about half that of their blood plasma.

My thanks are due to Dr E. Boomer of the Department of Chemistry for suggestions in the construction of the electrode vessel, and to Prof. Sackville of the Department of Animal Husbandry for access to the cows.

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637.127:543

126. NOTE ON THE SULPHYDRYL COMPOUNDS OF MILK

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In view of the wide distribution of glutathione, it is of some interest to observe that it is not present in milk. Its absence from this secretion is perhaps not surprising in the light of Hunter and Eagles'(1) observation that, though present in the blood cells it is absent from the plasma. The presence of free sulphydryl compounds in milk is also of importance in relation to the property possessed by milk of reducing methylene blue, a subject which has been given some attention in the succeeding paper (p. 31). Viale(2) reported a positive nitroprusside test in cow's and sheep's milk and considered the substance responsible for the test to be cysteine. Martini(3) claimed to have isolated glutathione from cow's and goat's milk.

Experimental

Nitroprusside tests were performed on a large number of milks, but in no case was there a positive test for the sulphydryl group. If, however, after saturation with ammonium sulphate, sodium cyanide was added and the milk allowed to stand a few minutes before adding the sodium nitroprusside, a faint but positive reaction occurred in all cases. This would suggest that the substance giving the test is probably in solution in the oxidised form and that it is reduced by the sodium cyanide. In which case it should be possible to precipitate the sulphydryl group according to the usual methods.

Protein-free filtrates of milk were prepared by precipitating the casein with *N* acetic acid and the lactalbumin and the lactoglobulin with heat. The resulting clear filtrate gave a negative biuret test. Sufficient ammonium hydroxide was added to precipitate the phosphates, and the supernatant fluid was then brought back to a faintly acid reaction and precipitated with a slight excess of lead acetate. The lead precipitate was decomposed by triturating with 0·2*N* sulphuric acid. Each step was followed by the nitroprusside test, with and without the addition of sodium cyanide, and while positive in the original sample treated with sodium cyanide, the nitroprusside test was negative in the protein-free filtrate and in each subsequent step. The active substance would thus appear to be either in or adsorbed to the protein. To determine the action of sulphydryl compounds in free solution in milk 1 mg. each of reduced glutathione and of cysteine hydrochloride in aqueous solution was added to two 20 ml. samples of milk. Both gave a nitroprusside test of greater colour intensity than that of milk treated with sodium cyanide. The samples were then subjected to the procedure outlined above.

Both substances remained in solution in the protein-free filtrates, were not adsorbed by the protein, were quantitatively precipitated by lead acetate, and were freed by decomposition of the lead precipitate with 0·2 *N* sulphuric acid.

It is well known that egg albumin, which normally gives no nitroprusside test, will do so when heated and thus denatured. If sodium cyanide is added to egg albumin, the latter is denatured and gives a nitroprusside test. In similar fashion an aqueous suspension of sodium caseinate will not give a nitroprusside test but on the addition of sodium cyanide will do so. Of the three principal proteins in milk, lactalbumin contains about 1·75 per cent. of cystine, while casein contains only about 0·25 per cent. Martini states that there is twice as much glutathione in goat's milk as there is in cow's milk. It is interesting to note that the lactalbumin content of cow's milk is about 0·5 per cent., while that of goat's milk is about 1·0 per cent. or nearly twice as much, while that of human milk is about 1·25 per cent.

Five ml. samples of the three different milks (cow, goat, and human) were saturated with ammonium sulphate, treated with 2 ml. 5 per cent. sodium cyanide, and the intensity of coloration observed on the addition of sodium nitroprusside. The comparative colour intensity of the three different milks and their protein percentages, according to associates of Rogers (4), are given in Table I.

Table I

	Cow	Goat	Human
Casein	2·95	3·04	0·91
Lactalbumin	0·52	0·99	1·23
Nitroprusside test (with sodium cyanide)	+	+ + +	+ + + +

The human milk[1] was 11 days post-partum and visibly free from colostrum. The goat's milk was from the mid-lactation period and the cow's milk a mixed vat sample.

SUMMARY

No sulphydryl compound in free solution could be found in cow's milk. It is suggested that the positive nitroprusside test obtained in milk treated with sodium cyanide is traceable to the cystine in the protein complex.

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[1] The human milk was obtained through the courtesy of the Obstetrical Department, The University Hospital.

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637.127.6

127. FACTORS IN THE REDUCTION OF METHYLENE BLUE IN MILK

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(With 3 Figures)

The reduction of methylene blue in milk has long been ascribed to bacterial action. Helmholtz(1) observed the reduction of litmus by bacteria. Neisser and Wechsberg(2) suggested that methylene blue might be used to determine the bacterial content of milk. Fred(3) compiled a comprehensive bibliography on the quantitative reduction of methylene blue by bacteria found in milk. The extensive use of the standard methylene-blue reduction test for grading market milk presupposes that the test is a measure of bacterial content.

On the other hand, Burri and Kursteiner(4) showed that reduction of methylene blue takes place in fresh milk in which bacterial action has been inhibited by antiseptics. Barthel(5) was led to conclude that the reduction of methylene blue in milk is dependent on two factors: (*a*) the removal of the dissolved oxygen by the growing bacteria and (*b*) the reduction of the dye by constituents of the milk. Later (Barthel(6)) he demonstrated rapid reduction of methylene blue in de-aerated fresh milk of low bacterial content, and in de-aerated sterile milk. He supposed the citrate in the milk to be the hydrogen donator and the action to be catalysed by the milk salts.

Clark, Cohen and Gibbs(7), in their extensive investigations on methylene blue, demonstrated its use as an oxidation-reduction indicator. Since methylene blue indicates only a comparatively narrow range of reduction intensity, they followed the oxidation-reduction potentials of milk by electrode measurement and suggested that further study along this line might give more detailed information of the reduction phenomenon. Thornton and Hastings(8, 9) found that methylene blue in milk reduced over a slightly higher potential range than that reported by Clark *et al.*, and attributed the result to salt effect or the influence of other oxidation-reduction systems. They showed that the typical potential range of milk is not appreciably affected by the addition of methylene blue in the dilution used in the test.

The original conception that methylene blue is reduced to the leuco form by the addition of two atoms of hydrogen must be modified to conform to the theory that oxidation-reduction processes are explicable in terms of electron migration. Michaelis' theory(10) of the reduction of the dye is the most attractive. This electronic concept provides a simple method (that of the electrode potential) for studying this reversible oxidation-reduction process in milk.

Viale(11) claimed that he demonstrated the presence of a sulphydril group in milk and assumed it to be responsible for the reduction. Schwarz(12) likewise attributed the reduction to sulphydryl systems produced by degradation of the proteins. Martini(13) claimed that he isolated glutathione from milk and believed that it becomes oxidised in the presence of sunlight and that methylene blue is reduced in the process.

Whitehead(14, 15) observed the catalytic effect of sunlight on the reduction of methylene blue in milk, and suggested that this was due to an oxidation-reduction reaction in which fats are oxidised and the methylene blue acts as a hydrogen acceptor. Whitehead's observations have been confirmed by Aikens and Fay(16) and by Frayer(17).

Experimental

All methylene-blue reduction tests, unless otherwise stated, were conducted according to the technique prescribed by *Standard Methods of Milk Analysis*(18). The constant temperature water-bath used in this work was electrically heated and thermostatically controlled. The temperatures used throughout were 37–38° C. In the electrometric work a type "K 2" Leeds and Northrup potentiometer[1] and a saturated calomel half-cell were used. Burnished platinum foil was used for electrodes. The oxidation-reduction potential measurements were made at 37–38° C. A new potassium chloride-agar bridge was used in each instance. The technique for obtaining anaerobic milk and measuring its potential in the absence of oxygen is described in the accompanying paper (p. 25).

Oxidation-reduction potentials

Under aerobic conditions.

The oxidation-reduction potentials of a number of milks were followed. The results are in close agreement with the potentials observed by other workers (see Fig. 1).

Under anaerobic conditions.

There appears to be no information available on the oxidation-reduction potentials of milk in the udder or of anaerobically drawn milk. It was observed that when milk is drawn into a partially exhausted tube, in which there is methylene blue, there is rapid reduction of the dye, the time taken for reduction varying from a few seconds to as many minutes. Milk drawn aerobically from the same source did not reduce the dye, in any particular case, in less than 8 hours. The milks were examined microscopically and appeared normal as far as numbers of bacteria and leucocytes can be considered as criteria. Measurements were made by the visual reduction of certain indicators and also electrometrically. Table I records the effect of anaerobically drawn milk on seven indicators.

[1] This apparatus was purchased with funds from the National Research Council of Canada.

Table I

Indicator	Mg. of dye in 10 ml. of milk	E_0 at *p*H 6·8 v.	Reduction time
m-Cresolindophenol	0·050	+0·221	30–40 sec.
o-Cresolindophenol	0·012	+0·204	Immediate
Thymolindophenol	0·200	+0·185	Immediate
Indo-2 : 6-dibromophenol	0·050	+0·135	30–40 sec.
Methylene blue	0·015	+0·017	1–30 min.
Potassium indigotetrasulphonate	0·100	−0·037	1–45 min.
Potassium indigotrisulphonate	0·100	−0·072	No reduction in 2 hours

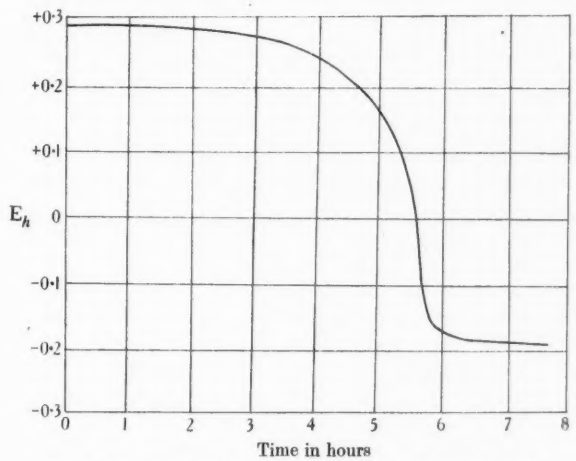

Fig. 1. The potential-time curve of a sample of market milk.

It will be seen from Table I that four of the indicators used, even in low concentration, over-poised the system, and that the time taken for the milk to come into equilibrium with the indicator varied from 30 sec. to 45 min. Thus it would appear that milk under anaerobic conditions, while having a fairly well-defined level of potential, is not well poised and has only a small oxidation-reduction capacity. The first indicator used, *m*-cresolindophenol, exhibited slight "poising," while the second indicator, *o*-cresolindophenol (which has a more negative E_0), reduced at once. This may be explained by the fact that the concentration of the first dye was four times that of the second.

Table II records the initial potential of five anaerobically drawn milks and the subsequent potentials after admitting air, determined by electrode potential measurement. The initial potentials obtained were more positive

Table II

Sample	Initial E_h v.	E_h after exposure to air v.
1	+0·1202	+0·2903
2	+0·1563	+0·2805
3	+0·1825	+0·3801
4	+0·1865	+0·2803
5	+0·1895	+0·2982

than might be expected from the indicator observations recorded in Table I. It is probable that the technique for transferring the sample from the container to the electrode vessel was not perfect. On admitting air, however, all of the potentials became immediately more positive, and in the range of fresh aerobically drawn milk. In observing the potentials of milk under aerobic conditions visual reduction of methylene blue took place when the E_h at the electrode was as high as +0·200, which confirms the findings of Thornton and Hastings(8) who report complete visual reduction in different milks at E_h values between +0·075 and +0·225 v. It is not uncommon to observe visual reduction in portions of a tube, while other parts show no sign of reduction. This would suggest that there are different reduction intensities in different parts of the sample. A simple experiment makes this evident. Four electrodes were placed in the same sample of milk to which methylene blue had been added and the potentials determined. The accuracy of the electrodes was previously determined against a standard phosphate buffer and showed a variance of not more than 0·0010 v. Table III records the results.

Table III

Time in hours	Electrodes			
	1	2	3	4
	E_h v.			
0	+0·2867	+0·2968	+0·2969	+0·2876
1	0·2560	0·2769	0·2662	0·2567
2	0·2493	0·2672	0·2532	0·2462
4	0·2390	0·2562	0·2500	0·2388
6	0·2360	0·2455	0·2224	0·2288
8	0·2220	0·2302	0·1928	0·1824
10	0·2000	0·2136	0·1129	0·1701
12	0·1019	0·0876	0·0654	0·0778
13	−0·1983	−0·1976	−0·2001	−0·1980

Examination of the table will show that quite a wide variation in potential occurred in different parts of the milk until the negative limit was reached, when there was approximate agreement. Visual reduction was complete at the end of the twelfth hour.

Influence of certain sulphydryl compounds, lactoflavin, and lactose on the reduction time

The absence from milk of a sulphydryl group in free solution has been reported in the accompanying paper (p. 29). However, the effect of the addition of certain sulphydryl compounds on the methylene-blue reduction times and on the oxidation-reduction potentials in milk was observed.

Cystine.

The addition of 5 mg. cystine to 10 ml. milk had no effect on either the methylene-blue reduction times or the potentials.

Cysteine hydrochloride.

Table IV shows the effect on the methylene-blue reduction times of the addition of 5, 2 and 1 mg. cysteine hydrochloride to 10 ml. milk.

Table IV

Sample	Reduction time Hours	Min.	Sample	Reduction time Hours	Min.
Control A	9	40	A + 2 mg. cysteine	1	45
	9	30		1	45
Control B	8	30	B + 2 mg. „	1	30
	8	30		1	35
A + 5 mg. cysteine	0	45	A + 1 mg. „	2	30
	0	45		2	30
B + 5 mg. „	0	40	B + 1 mg. „	2	15
	0	50		2	15

Fig. 2 illustrates the potential-time curve of a sample of market milk with and without the addition of 5 mg. cysteine hydrochloride.

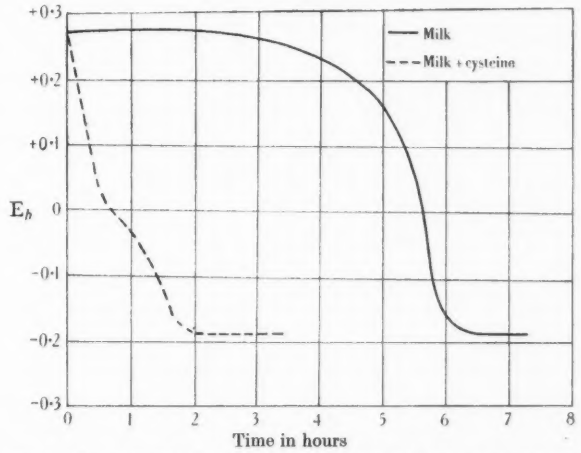

Fig. 2. The potential-time curves of a market milk with and without the addition of cysteine.

Glutathione.

Table V shows the effect on the methylene-blue reduction times of the addition of 5, 2 and 1 mg. reduced glutathione to 10 ml. milk.

Table V

Sample	Reduction time Hours	Min.	Sample	Reduction time Hours	Min.
Control A	7	30	A + 2 mg. glutathione	2	50
	7	30		2	55
Control B	8	15	B + 2 mg. „	3	00
	8	20		2	50
A + 5 mg. glutathione	1	00	A + 1 mg. „	4	10
	0	55		4	00
B + 5 mg. „	1	00	B + 1 mg. „	4	25
	1	00		4	25

Fig. 3 illustrates the potential-time curve of a sample of market milk with and without the addition of 2 mg. cysteine hydrochloride and 2 mg. reduced

glutathione respectively. It should be noted that the glutathione has approximately half as many —SH groups as are present in the cysteine, thus the difference in time between the drop in potential of the two samples is explicable.

Ergothioneine.

No ergothioneine could be found in milk according to Hunter's[19] diazo test. The addition of 5 mg. ergothioneine to 10 ml. milk caused no change in the methylene-blue reduction times or in the oxidation-reduction potentials.

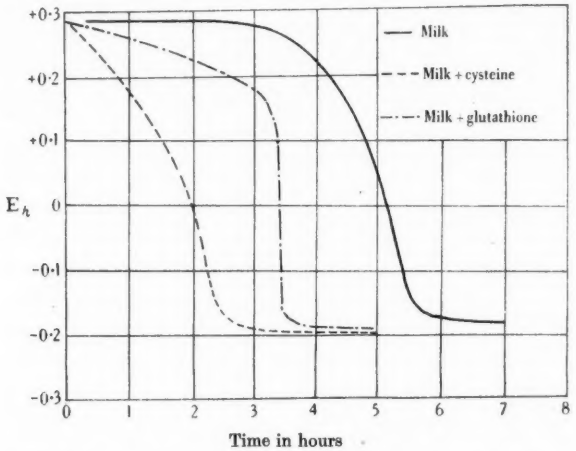

Fig. 3. Potential-time curves of a market milk with and without the addition of cysteine and glutathione.

Lactoflavin.

The isolation from whey of lactoflavin (Kuhn and Wagner-Jauregg[20] and Ellinger and Koschara[21]), a highly reversible redox system that is photochemically active, suggested that it might be a factor in the methylene-blue reducing system of milk. A method for its preparation, based on the procedure of the workers mentioned above, was followed. Ten litres of whey were shaken for 2 hours with 240 g. kaolin. The kaolin was taken off in about a litre of the whey which was centrifuged till clear. The sediment was washed with water until the washings were free from lactose and then eluted by shaking for 1 hour with a mixture of 270 ml. 85 per cent. methyl alcohol, 270 ml. pyridine and 1080 ml. water and allowing to stand overnight in a refrigerator. It was then centrifuged and the supernatant fluid concentrated *in vacuo* to about 200 ml. An equal volume of 85 per cent. methyl alcohol was added to precipitate the colloidal kaolin. The final aqueous extract was washed twice with ether to remove carotinoid pigments and a litre of acetone added to precipitate lactose. The filtrate was then concentrated *in vacuo* to about 50 ml. and was again treated with acetone and re-evaporated to a volume of about 10 ml. During the preparation care was taken to exclude as much light as possible. The final aqueous solution (*p*H 5·0) was a clear yellow colour with

a green-gold fluorescence. It contained lactose and gave a positive test with Folin's uric acid reagent, due at least partially to the presence of uric acid. The effect of the addition of this extract on the methylene-blue reduction times of milk are recorded in Table VI.

Table VI

Sample		Reduction time	
		Hours	Min.
Control	No reduction in	7	00
,, +0·05 ml.		0	30
,, +0·10 ml.		0	30
,, +0·15 ml.		0	25
,, +0·20 ml.		0	25

Four days after preparation the extract became inactivated and had no effect on methylene-blue reduction times when added in large quantities. A second extract was prepared from a large quantity of whey (175 litres) and was a week in preparation. Table VII records the methylene-blue reduction times of samples of market milk with and without the addition of varying quantities of the extract.

Table VII

Sample		Reduction time	
		Hours	Min.
Control	No reduction in	7	00
,, +0·05 ml.		2	30
,, +0·10 ml.		2	00
,, +0·15 ml.		1	30
,, +0·20 ml.		1	30

On the second day after preparation this extract lost its activity and would not hasten reduction of methylene blue in milk. Attempts to restore the activity of the extract by reducing it in alkaline solution with hydrogen sulphide or sulphur dioxide were unsuccessful.

The fact that an extract can be prepared from whey which catalyses the reduction of methylene blue in milk warrants further investigation.

Lactose.

The addition of lactose to milk in varying quantities had no effect on methylene-blue reduction times, nor would aqueous lactose solutions reduce methylene blue under the conditions of the test. If the *p*H of an aqueous solution of lactose was adjusted to over 10·0, methylene blue was reduced. Since the *p*H of milk is fairly constant, the extreme ranges under normal conditions being between 6·4 and 7·2, it would seem improbable that lactose plays any direct part in the reduction of methylene blue in milk.

The effect of light

The effect on the methylene-blue reduction times of the addition of cysteine, reduced glutathione, and lactose to milk exposed to varying conditions of light was observed. The addition of lactose had no effect on the reduction times. Table VIII records the methylene-blue reduction times of duplicate samples

of two market milks (A and B) with and without the addition of cysteine: (1) exposed to sunlight through glass; (2) placed in the dark; (3) in a constant temperature water-bath in diffuse light.

Table VIII

Sample	(1) Exposed to sunlight, temp. 26·6°C. Hours	Min.		(2) Placed in dark, temp. 23·5°C. Hours	Min.	(3) In water-bath, temp. 37·5°C. Hours	Min.
Control A	0	35	No reduction in	24	00	10	00
	0	35	,,	24	00	10	20
Control B	0	45	,,	24	00	9	45
	0	45	,,	24	00	10	00
A + 5 mg. cysteine	0	05		2	30	0	45
	0	05		2	25	0	50
B + 5 mg. ,,	0	05		1	50	0	50
	0	06		2	00	0	55
A + 1 mg. ,,	0	08	No reduction in	24	00	2	35
	0	08	,,	24	00	2	40
B + 1 mg. ,,	0	10	,,	24	00	2	00
	0	10	,,	24	00	2	10

It will be seen from this table that, of the samples placed in the dark, only those to which 5 mg. cysteine were added reduced. The addition of 1 mg. of cysteine was not sufficient to cause a fall in potential through the range of methylene blue. The exceedingly strong catalytic effect of sunlight in the presence of cysteine is evident. The experiment was repeated using reduced glutathione and similar results were obtained.

Discussion

The present paper is in the nature of a survey of this very old problem. The experimental results would appear to alter appreciably current conceptions with regard to the factors at play in milk leading to the reduction of methylene blue. The want of any strict parallelism between bacterial count or leucocyte count and methylene-blue reduction times, recorded from time to time in the literature (Thornton *et al.* (22)), and observed by the writer in the routine testing of market milks, has led him to suspend judgment on the influence of organised elements in milk on the methylene-blue reduction test. If we can assume that we are dealing with only one mechanism—and we have no evidence for more—then the finding reported here that anaerobically drawn milk reduces methylene blue instantaneously alone suffices to prove that neither bacteria nor leucocytes necessarily play any direct part in that mechanism. The usefulness of the test for the grading of market milks is not, however, thereby impugned, as our experience indicates that some degree of de-oxygenation of the milk is necessary before reduction can take place—a condition which bacteria may well bring about in the usual practical application of the test. It is not our object here to assess the importance of bacteria in the reduction of methylene blue when grading milk for market purposes, but it appears from the literature cited, and results reported here make it

evident, that bacterial action has no necessary relation to the reduction phenomenon. There is in milk, quite independent of organised constituents, a substance or redox system which in itself rapidly reduces methylene blue.

The observation that milk drawn anaerobically from the udder reduces methylene blue almost instantaneously provides a partial clue to the mechanism of reduction. The behaviour of such milks towards oxidation-reduction indicators and the measurement of their oxidation-reduction potential before and after exposure to air establishes the presence in the milks of a redox system with a well-defined level of potential which is negative to the potential of milk which has been exposed to oxygen. Milk, as it exists in the udder, shows a potential which is negative to methylene blue, but when exposed to oxygen the potential immediately rises to a point where it is positive to the reduction range of methylene blue on the E_h scale, and so the dye is quickly reduced or remains unreduced respectively.

The further deduction from the small oxidation-reduction capacity that the substance or substances composing this redox system are present in small amount is suggestive that the major constituents of the milk, protein, fat and sugar, play no part in the mechanism.

Before the writer had satisfied himself that no free sulphydryl compounds are present in milk, the effects of cysteine and reduced glutathione on the oxidation-reduction potentials had been studied as recorded. An almost perfect explanation of the test appeared to be at hand. Small amounts of either substance added to milk greatly increases the rate of reduction of methylene blue, the effect is enhanced by exposure to light, and the oxidation-reduction potential curves obtained with the addition of cysteine or glutathione are substantially similar to those of milk without such addition. Yet the writer has since satisfied himself that neither cysteine nor reduced glutathione is present in sufficient quantities even in anaerobically drawn milk to bring about the reduction of methylene blue.

The writer's experience with lactoflavin can be regarded as nothing more than a suggestion for further investigation. There was no opportunity to prepare or obtain the crystalline material, without which any findings in this regard must remain inconclusive. The known sensitivity of lactoflavin, its presence in milk probably in such amount as to lead one to believe the reducing substance is present, and the positive findings above recorded with solutions certainly containing the material, would suggest that this possibility should be investigated before proceeding further.

SUMMARY

1. Bacteria may play but an insignificant part in the reduction of methylene blue in milk, though their de-oxygenating effect may be of influence in the commercial application of the test.

2. Milk as it exists in the udder, or milk drawn anaerobically, reduces

methylene blue almost instantaneously, whereas the same milk exposed to oxygen will usually take more than 10 hours to reduce.

3. The oxidation-reduction potential of anaerobically drawn milk is much lower than the same milk exposed to oxygen, and in accordance with its behaviour toward methylene blue.

4. Evidence is given for the presence of a redox system, present in low concentration, as responsible for the reduction of methylene blue.

5. Although the addition of small amounts of cysteine or glutathione to milk leads to the reduction of methylene blue, their absence from milk excludes them as possible factors in the normal reduction.

6. The possibility that lactoflavin may furnish the redox system is suggested.

7. The reduction of methylene blue in milk is catalysed by light in the visible spectrum.

The writer desires to express his thanks to Prof. G. Hunter who suggested this research and has throughout it lent both guidance and criticism. He is also greatly indebted to Prof. H. R. Thornton of the Department of Dairying for the close co-operation rendered possible with that Department, for assistance in the supply of milks, and for the use of various parts of equipment. Finally, the writer would express his indebtedness for financial assistance to the Milk Industries of Alberta.

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128. THE DETERMINATION OF LACTOSE AND GLUCOSE IN MILK

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(With 1 Figure)

WHITNAH(1, 2) observed that, on treating deproteinised milk with washed baker's yeast, there was a decrease in the dextro-rotation, which for certain reasons he attributed to glucose. He later found that the concentration of this glucose was greater in the milk of cows whose blood-sugar concentration had been increased by forced feeding with glucose solution. The work described in this paper was carried out in order to confirm this observation and extend its scope to include as many types of milk as were available.

For the determination of reducing substance the method of Somogyi(3) (reagent 2), in conjunction with the van Slyke and Hawkins(4) method of protein precipitation, was chosen because of its specificity in the presence of non-sugar-reducing substances, which are likely to occur in milk. The tungstic acid filtrates were treated with washed yeast as in Whitnah's experiments.

The present work has shown that reducing material can be determined with great precision by the application of Somogyi's method. Except with freshly washed yeast, no removal of lactose takes place and the yeast adds no reducing material to the milk filtrates. Glucose added to milk in concentrations of 0·2–1·0 per cent. is removed quantitatively by the yeast, and the increased sugar is determined with a good degree of accuracy considering the lactose : glucose ratio of the sugar present.

For the sake of brevity, the reducing material removable by washed yeast at a neutral or slightly acid reaction is termed glucose, since glucose is the only common dextro-rotatory reducing substance, removable by yeast, which is likely to be present in milk.

EXPERIMENTAL

The determination of reducing sugar

Reagents.

(1) Somogyi's reagent. This is prepared according to Somogyi's directions.

(2) Pure benzene.

(3) 0·005 N sodium thiosulphate. This is prepared freshly each day from 0·1 N thiosulphate, which is preserved by the presence in solution of 0·5 per cent. amyl alcohol. This does not interfere with the determination, and the thiosulphate remains clear and preserves its strength for several months. The thiosulphate is standardised daily by means of the Somogyi reagent, which

contains KI and KIO_3, and on acidification of 5 ml. yields a quantity of iodine equivalent to 22·0 ml. of 0·005 *N* thiosulphate. The reagent is originally standardised by titration of a convenient quantity, after acidification, with the 0·1 *N* stock solution of thiosulphate.

(4) 1 per cent. soluble starch in saturated sodium chloride.

(5) 2 *N* sulphuric acid.

Method.

The determinations are carried out, in batches of a dozen or so, using 7 × 1 in. boiling tubes, loosely stoppered by thin glass bulbs 1·2 in. in diameter. By means of calibrated Ostwald pipettes, 5 ml. of the Somogyi reagent and 5 ml. of the sugar solution are measured into each boiling tube. After addition of 2–3 drops of pure benzene, the contents are mixed by lateral rotation and the stoppers placed in position. The tubes are placed in a rapidly boiling water-bath for exactly 15 min., after which they are immediately cooled in cold running water. Care must be taken to avoid agitation of the contents of the tubes while cooling in order to avoid re-oxidation of the cuprous oxide by indrawn atmospheric oxygen. When cool, the contents of the tubes are acidified with gentle stirring with 2·5 ml. of 2 *N* sulphuric acid, taking care that the white precipitate of cuprous iodide is dissolved. The iodine in excess of that required to oxidise the cuprous oxide is now titrated with the 0·005 *N* sodium thiosulphate using starch when nearing the end-point. Blank determinations in which distilled water is used in place of the sugar solution are carried out at the same time. It sometimes happens, especially when relatively large amounts of sugar, with consequently small thiosulphate titres, are being determined, that the concentration of acid is so great that some thiosulphate is decomposed giving a cloudy suspension which prevents the accurate determination of the end-point. Dilution of the contents of the tube with an equal quantity of water before titration obviates this difficulty.

The difference between the titres of the blank and the actual determination represents the amount of iodine used in oxidising the quantity of cuprous oxide formed by the sugar present in 5 ml. of the solution. This varies from sugar to sugar, and curves can be constructed experimentally to show the relationship between the amount of the particular sugar and the iodine needed to oxidise the cuprous oxide under the conditions specified above or, alternatively, the thiosulphate used to decolorise the iodine. Such a curve is given by Somogyi(3) for glucose. A similar curve which has been constructed for lactose is shown in Fig. 1 (using the upper scale of abscissae).

This curve is represented by the equation:

$$\text{mg. lactose in 5 ml.} = 0{\cdot}23\ (\text{ml. } 0{\cdot}005\,N \text{ thiosulphate}) + 0{\cdot}05. \quad \ldots\ldots(1)$$

Like most thiosulphate-sugar curves, it does not quite pass through the origin, and the thiosulphate is therefore not exactly proportional to the sugar concentration.

Determination of total reducing sugar as lactose in milk

Reagent.

10 per cent. aqueous sodium tungstate (Analar), 1 vol.; 0·667 *N* sulphuric acid, 1 vol.

Mix and dilute immediately to nine volumes. Make up freshly each day.

One volume of milk (preferably 5 or 10 ml.) is placed in a flask graduated at ten times the volume and diluted to the mark with constant mixing with the above reagent. After a few minutes the mixture is filtered through a Whatman No. 40 filter, previously washed several times with water and dried. A portion of the filtrate, which should be prefectly clear, is diluted to ten times its volume, thus making a 1 : 100 dilution in all. Five ml. portions of the dilution are analysed as above. The lactose concentration is then calculated from equation (2) or read from the graph (Fig. 1), using the lower scale of

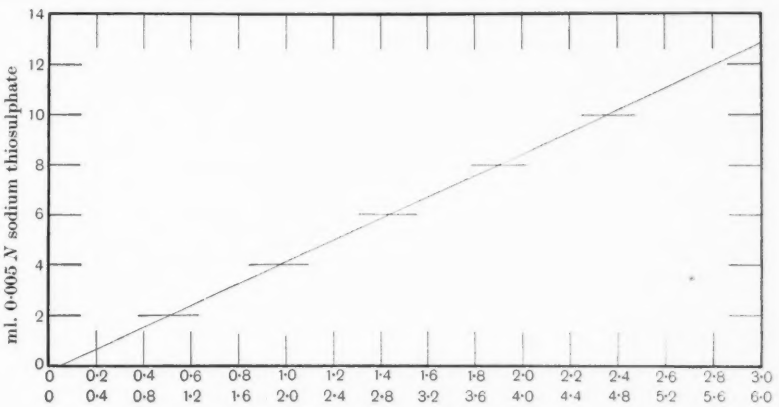

Fig. 1. Upper scale: mg. lactose in 5 ml. solution.
Lower scale: per cent. lactose in milk (dilution 1 : 100).

abscissae. The amount of lactose in 5 ml. solution is given by equation (1), and this equation transformed for milk at 1 : 100 dilution takes the form:

$$\text{per cent. lactose} = 0{\cdot}46\ (\text{ml. } 0{\cdot}005\,N \text{ thiosulphate}) + 0{\cdot}1. \quad \ldots\ldots(2)$$

The precision of this method is illustrated by the following experiment. Ten-ml. samples of milk were measured into eight 100 ml. standard flasks and made up to the mark with tungstic acid solution and filtered. Ten-ml. portions were further diluted to 100 ml. Duplicate 5-ml. portions were taken for determination of the total reducing sugar. At the same time ten determinations of the blank using distilled water were made. The blanks gave a mean of 20·20 ml. with a standard error of the mean $\pm 0{\cdot}002$, which is 0·01 per cent. of the mean. The mean titre of the sugar determinations was 10·77 for the sixteen determinations with a standard error of the mean of $\pm 0{\cdot}039$, which is 0·36 per cent. of the mean.

The determination of glucose and lactose in milk

Reagent.

Commercial baker's yeast is washed on the centrifuge with three times its volume of water until the supernatant liquid is clear and free from reducing substances. Five or six washings are usually necessary. The yeast is finally made up to a 25 per cent. suspension, and stored in the refrigerator at 0–4° C., at which temperature it retains its glucose-removing properties for at least 10 days. The yeast should, for reasons given below, be stored for not less than 24 hours before use.

Method.

The 1 : 100 dilution of deproteinised milk is used. An amount of yeast suspension sufficient to yield 1 g. of yeast for each determination, with 1 g. extra for a blank, is taken and centrifuged at 3000 r.p.m. for 12 min. The precipitate is made up to 25 ml. for every determination and distributed in centrifuge tubes. After centrifuging for 12 min., the water is poured off and any moisture adhering to the inside of the tube is removed by means of filter paper. Twenty-five-ml. portions of the 1 : 100 dilutions are now added to the yeast in the tubes and mixed with the yeast by means of glass rods. A tube containing 25 ml. water in place of the deproteinised milk is put up at the same time. The tubes are placed in the incubator at 37° C. for 1 hour, the yeast centrifuged down and the supernatant liquid treated exactly as for a lactose determination. Determinations of the total reducing sugar in the milk are carried out at the same time. The difference between the thiosulphate titres of the blank, using yeast, and the unknown after-yeast treatment corresponds to the true *lactose* content of the milk. The corresponding difference between the titre for total reducing sugar and that for true lactose gives the volume of thiosulphate corresponding to the *glucose* present. The relation between the percentage of glucose in the milk and 0·005 N thiosulphate was found to be 1 ml. 0·005 N thiosulphate $\equiv$ 0·224 per cent. glucose in milk, in actual determinations of glucose added to milk. This compares favourably with Somogyi's figure of 0·226 per cent. That no reducing material is added to the solution by the washed yeast is shown by the two following sets of determinations of blanks: Blank using distilled water, mean 20·20 ml. 0·005 N thiosulphate, standard error of the mean $\pm$ 0·002 = 0·01 per cent. of mean (ten determinations). Blank using distilled water after yeast treatment 20·23 ml. 0·005 N thiosulphate, standard error of the mean $\pm$ 0·005 = 0·025 per cent. of mean (eleven determinations).

The three experiments illustrated in Table I show that yeast which has been washed and used at once removes 5 per cent. of the lactose added, while after being kept 24 hours or more in the refrigerator it removes none. The experiments were carried out under identical conditions, on similar solutions of pure lactose. The reducing material in the number of identical portions of

lactose solution shown in Table I was determined in duplicate before and after treatment with yeast. The figures are actual titres with the appropriate blanks shown. Exps. I, II and III were carried out on successive days, the yeast having been washed on the same day as Exp. I.

The two experiments given in Table II illustrate the completeness of removal of glucose added to milk in concentrations of from 0·2 to 1·0 per cent. and also provides the data for calculating the glucose equivalent to a given volume of thiosulphate. It is evident that all glucose added to milk is removed

Table I. *The yeast treatment of lactose solutions*

	Before yeast treatment			After yeast treatment			
Exp.	No. of determinations	Mean and standard error of mean ml.	Mean titre equivalent to sugar ml.	No. of determinations	Mean and standard error of mean ml.	Mean titre equivalent to sugar ml.	Loss or gain of lactose, equivalent to ml.
I	5	9·85 ±0·014	10·49 ±0·05	6	10·40 ±0·096	9·95 ±0·11	0·54 ±0·12
	Blank	20·34 ±0·05	—	Blank	20·35 ±0·05	—	=5 % loss
II	4	10·28 ±0·02	10·01 ±0·06	4	10·28 ±0·05	10·01 ±0·07	0·0 ±0·09
	Blank	20·29 ±0·06	—	Blank	20·29 ±0·05	—	=no loss
III	5	10·25 ±0·02	9·99 ±0·03	6	10·41 ±0·01	9·95 ±0·02	0·04 ±0·04
	Blank	20·24 ±0·02	—	Blank	20·36 ±0·02	—	=no loss

Table II. *Removal of glucose by yeast*

	Before yeast treatment				After yeast treatment				Difference in thiosulphate titre, before and after yeast treatment, equivalent to 0·1 % glucose	
mg. glucose per 100 ml. milk added	ml. thiosulphate	Difference blank and unknown ml.	Difference for added glucose	Difference for 0·1 %	ml. thiosulphate	Difference blank and unknown ml.	Difference for added glucose	Mean difference	Difference ml.	Mean difference and standard error of mean
0	10·60	9·60	—	—	11·28	8·92	—		—	
200	9·70	10·50	0·90	0·45	11·32	8·88	−0·04		0·47	
300	9·18	11·02	1·42	0·47	11·15	9·05	+0·13	0·09 ±0·06	0·43	0·45 ±0·009
500	8·52	11·68	2·08	0·42	11·43	8·77	−0·15		0·45	=0·45 ±2·0 %
1000	6·05	14·15	4·55	0·46	11·31	8·89	−0·03		0·46	
Blank	20·20	—	—	—	20·20	—	—		—	
0	11·56	8·66	—	—	12·14	8·15	—		—	
200	10·59	9·63	0·97	0·49	11·99	8·30	−0·15		0·41	
300	10·11	10·11	1·45	0·48	12·13	8·16	−0·01	0·06 ±0·05	0·48	0·44 ±0·015
500	9·28	10·94	2·28	0·46	12·08	8·21	−0·06		0·44	=0·44 ±3·4 %
1000	7·36	12·86	4·20	0·42	12·11	8·18	−0·03		0·42	
Blank	20·22	—	—	—	20·29	—	—		—	

by the washed yeast, any difference between the titre for milk with and without added glucose being not significantly different from the standard error of the determination. The last two columns of Table II show the volume of thiosulphate equivalent to 0·1 per cent. of added glucose, determined by the yeast method. The standard error in the two experiments is 2·0 and 3·4 per cent., and of the eight determinations taken as a whole is 1·8 per cent. Thus, although the glucose figures are the differences between two relatively large determinations, whose error is 0·36 per cent. and which have a fairly large inherent likelihood of error, small amounts of added glucose may be determined with an average error of 3 per cent. Therefore the likelihood that the substance

being considered is really glucose and that it is being determined with some degree of reliability is increased. The volume of 0·005 *N* thiosulphate equivalent to 0·1 per cent. glucose in milk is 0·445 ± 0·008 ml.

Table III shows some typical results obtained by these methods.

Table III

Cow	Total reducing sugar %	Lactose %	Glucose %	Remarks
1	4·52	4·36	0·08	Normal cows
2	4·62	4·41	0·10	,,
3	4·64	4·54	0·05	,,
4	4·50	4·46	0·02	,,
5	4·40	4·53	0·06	,,
6	4·71	4·67	0·04	,,
26	4·61	4·47	0·07	,,
27	4·20	3·98	0·11	Late lactation
18	3·77	3·55	0·11	,,
19	3·84	3·60	0·12	,,
14	3·85	3·55	0·15	,,
30	4·31	4·07	0·12	,,
29	4·27	4·09	0·09	,,

Summary

1. The application of the method of Somogyi to the determination of the reducing sugar in milk, with a precision of ± 0·36 per cent., is described.

2. Washed yeast does not ferment lactose and does not add reducing material to tungstic acid filtrates.

3. The reducing substance in milk removable by yeast can be determined with an accuracy of ± 3 per cent.

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129. THE CHLORINE CONTENT OF MILK AS AN INDICATION OF MASTITIS

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In recent years considerable attention has been directed to the high incidence of streptococcal mastitis in our dairy herds, and a method of control and elimination of the disease has been described by Minett *et al.*(1). The method suggested is based on the diagnosis of the disease by bacteriological methods, the segregation of the infected from the healthy animals, and milking them last. A similar system of controlling the disease has been suggested also by Plastridge *et al.*(2).

The cultural examination of samples of milk from individual quarters of the udder by the method described by Minett *et al.*(3), even when using the selective medium, crystal violet aesculin blood agar, of Edwards(4) proves rather laborious, and the substitution of a simpler method of diagnosis would considerably facilitate the adoption of the segregation method of controlling the disease on a large scale, provided, of course, it could be shown to be reasonably reliable. In view of a claim made by Rosell(5) that the chlorine content of milk was a better guide for the detection of mastitis than any other single test, and in view of the simplicity of the estimation recommended by him, it was decided to determine whether a simple chemical test of this description could be utilised with any degree of reliability. This worker points out that the chlorine content of normal milk lies between 0·08 and 0·12 per cent., and claims that any sample of milk containing more than 0·142 per cent. of chlorine, *i.e.* requiring the equivalent of more than 4 ml. 0·1 *N* silver nitrate, was a definite indication of the presence of mastitis.

A small herd, in which considerable trouble with chronic mastitis had been experienced and to which the segregation method had been recently applied for the purpose of controlling the disease, was selected for the investigation.

History of the herd

The milking herd of 25–30 cows has been maintained partially by rearing heifers on the farm, but mainly by the purchase of cows in the open market. Trouble with mastitis was first experienced early in 1934, and at that time the herd was being milked by machine. In August 1934 the use of the machine was discontinued, but considerable trouble with mastitis was still experienced during the next 6 months, when the cows were hand milked. During the latter part of 1934 any cows showing clinical symptoms of the disease were immediately segregated to a separate shed and milked last. Trouble was still experienced, and fresh cases among the apparently healthy group were rather

frequent. In March 1935 "quarter" samples were examined bacteriologically with a view to applying the segregation method (Minett *et al.*(1)) of control to the herd. At this time the apparently healthy group comprised nineteen animals, but there were still on the farm other animals showing clinical symptoms of mastitis in one or more "quarters", such animals being milked last.

Bacteriological diagnosis

The milk from each "quarter" was tested in the cowshed with brom-cresol purple test papers and, after washing the udders with chlorinated water and discarding the first stream of milk from each teat, "quarter" samples were drawn into sterile tubes of 20 ml. capacity. Ten ml. amounts were centrifuged, using special tubes similar to those described by Minett *et al.*(3), and the character and amount of deposit noted. Two saline dilutions of sediment were plated using aesculin crystal violet blood agar. The streptococci appearing on the plates were all weakly haemolytic, showing narrow zones of partial clearing around the colonies. Representative colonies on plates from different "quarters" were picked to tubes of broth and, after purification, their morphological appearance, action on litmus milk, containing methylene blue to a concentration of 1/20,000, sodium hippurate, lactose, saccharose, salicin, mannite, inulin, raffinose and final *p*H in glucose broth, respectively, were tested.

The chloride test

The method advocated by Rosell(5, 6) consists of the direct titration of milk with standard silver nitrate solution using potassium chromate as an internal indicator. Rosell used 10 ml. of milk diluted with 40 ml. distilled water to which were added 8–10 drops of a 10 per cent. solution of potassium chromate, the titration being carried out with 0·1 *N* silver nitrate.

The direct titration of chloride in milk—without the removal of protein—was suggested as long ago as 1909 by Richmond(7), who used a method based upon this principle for the detection of added salt in milk. In later years Hammer and Bailey(8) suggested a similar method for determining the approximate chlorine content of milk, and still later this method was used by Sharpe and Struble(9). In both these papers the authors noted that the percentages of chlorine found were always uniformly high. This led Sharpe and Struble to refer to the amount of chlorine found as the "direct titratable chloride value". Other workers who tried this method of direct titration were Poetschke(10, 11) and van der Berg and Koppejan(12). In these references it states that errors were due to casein in the milk, and that possible variations in the results obtained could be attributed to "differences in protein". A rather important reference to the direct titration method is to be found in a paper by Hucker(13). This worker criticised the method owing to the difficulty which he experienced in obtaining a satisfactory end-point. This criticism by Hucker was confirmed by the writers when the above method was applied to the samples under investigation. Only after matching the colour of the sample, being titrated,

against a standard coloured milk (a pale peach colour) could one obtain reasonable agreement between duplicate determinations. It should be noted here that the advocates of the method suggest that the end-point is reached only when a deep orange colour is produced. When titrations were carried to this stage, agreement could not be obtained between duplicate determinations. Moreover, the percentage of chlorine found was always absurdly high. Apparently the chief cause of error was the unusually dense granular precipitate of silver chromate which appeared in the liquid immediately all chloride had been precipitated, the precipitate being entirely masked by the curd separating from the milk. Even after violent agitation the red colour of the silver chromate could be recognised only when there was a considerable quantity of this compound present, *i.e.* following the addition of a large excess of silver nitrate.

In attempting to modify the procedure it was found that by increasing the amount of indicator to 2 ml. a more satisfactory end-point could be obtained; furthermore, by diluting the milk with 80 ml. of water and adding 2 ml. of potassium chromate as indicator, the colour change was well defined. These observations are in accordance with those of Sharpe and Struble (9). The percentage of chlorine found when using these modifications was considerably lower than that corresponding to the titration figure obtained when using the original method. Even so, assuming that all samples with a chlorine content of over 0·14 per cent. are obtained from the milk of cows suffering from mastitis, a considerable number of cows and even heifers gave positive results by this test which were negative on cultural examination. Owing to these unsatisfactory results it was decided to compare the method advocated by Rosell with methods involving the removal of protein from the milk before the chlorine is determined.

Experimental

The chlorine content of six 10-ml. portions of well-mixed milk was determined by each of the following methods:

(*a*) The method advocated by Rosell (5, 6), *i.e.* by diluting 10 ml. milk with 40 ml. of water, adding 10 drops of potassium chromate as indicator and titrating with 0·1 *N* silver nitrate.

(*b*) Modification of method (*a*), *i.e.* diluting 10 ml. milk with 80 ml. water, adding 2 ml. of potassium chromate as indicator and titrating with 0·1 *N* silver nitrate.

(*c*) Method of Austin and van Slyke (14), whereby the protein is precipitated with picric acid and, after filtering, the chlorine is determined by Volhard's method.

(*d*) Ashing the milk at low temperature and determining the chlorine in the ash.

(*e*) A wet digestion method of Davies (15), in which the milk is heated on a water bath with concentrated nitric acid after the addition of an excess of 0·1 *N* silver nitrate. After the solution has become clear, the excess silver

nitrate is titrated with 0·1 *N* potassium thiocyanate, using a solution of ferric alum acidified with nitric acid, as indicator.

The titration figures are given in Table I and the percentage of chlorine in Table II.

Table I. *ml.* 0·1 *N silver nitrate per* 100 *ml. milk*

	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)
	3·15	2·60	2·22	2·15	2·20
	3·10	2·60	2·20	2·15	2·17
	3·00	2·65	2·22	2·17	2·20
	3·05	2·70	2·17	2·10	2·20
	3·00	2·60	2·20	2·15	2·15
	2·90	2·70	2·17	2·15	2·17
Mean	3·033	2·642	2·197	2·145	2·182

Table II. *Grams chlorine per* 100 *ml. milk*

	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)
	0·112	0·092	0·079	0·076	0·078
	0·110	0·092	0·078	0·076	0·077
	0·106	0·094	0·079	0·077	0·077
	0·108	0·096	0·077	0·074	0·078
	0·106	0·092	0·078	0·076	0·076
	0·102	0·094	0·077	0·076	0·077
Mean	0·107	0·093	0·078	0·076	0·077

It will be observed that method (*a*) is the only one which failed to give reasonably concordant results. In spite of the close agreement of the results obtained by the modified direct titration method (*b*), these are all consistently higher (approximately 20 per cent.) than the results obtained by methods (*c*), (*d*) and (*e*), in which the protein is removed or destroyed before the estimation of chlorine is commenced. The last three methods gave mean chlorine percentages which are comparable, though that derived by method (*d*) is slightly lower, due to a slight loss by volatilisation during ashing. To subject the direct titration methods to a more extended test it was decided to submit the samples of milk from the herd in question to analysis by methods (*a*), (*b*) and (*e*).

Quarter samples of milk from the nineteen apparently healthy cows were examined by the methods previously described under bacteriological diagnosis. The results of the chlorine determination by the three methods (*a*), (*b*) and (*e*), together with the results of bacteriological examination of "quarter" samples of milk from these cows, are given in Table III. The figures for seven of the cows have been omitted from the table, as the cultural examinations of "quarter" samples were negative and the chlorine content of the milk fell below 0·14 per cent. by each of the three methods.

Discussion

An examination of the data for chlorine shows that in every sample the ratios between the amounts found by each of the three methods are similar, *i.e.* methods (*a*) and (*b*) gave results approximately 25 and 15 per cent. higher,

Table III. *Grams chlorine per* 100 *ml. of milk*

		Samples taken April 3			Samples taken April 15			Bacteriological observations*	
		Method (*a*)	Method (*b*)	Method (*c*)	Method (*a*)	Method (*b*)	Method (*c*)	Samples taken March 7	Samples taken April 15
Barnet	R.F.	0·140	0·131	0·113	0·135	0·121	0·106		+
	L.F.	0·174	0·158	0·138	0·215	0·190	0·180	Bulk	+
	R.H.	0·138	0·128	0·110	0·145	0·128	0·099	+	+
	L.H.	0·167	0·147	0·138	0·170	0·142	0·130		+
Red Limb	R.F.	0·149	0·140	0·131	0·163	0·142	0·116	+	+
	L.F.	0·138	0·128	0·106	0·129	0·108	0·091	+	+
	R.H.	0·136	0·128	0·106	0·128	0·110	0·087	+	+
	L.H.	0·131	0·118	0·092	0·128	0·106	0·085	−	−
Wissendine	R.F.	0·149	0·135	0·117	0·151	0·131	0·109	−	−
	L.F.	0·255	0·236	0·209	0·227	0·209	0·187	−	−
	R.H.	0·151	0·135	0·124	0·145	0·124	0·105	−	−
	L.H.	0·138	0·122	0·108	0·124	0·106	0·082	+	+
Morris	R.F.	0·156	0·144	0·124	0·223	0·206	0·190	−	−
	L.F.	0·140	0·134	0·115	0·149	0·129	0·099	−	−
	R.H.	0·191	0·167	0·156	0·280	0·262	0·243	−	−
	L.H.	0·136	0·124	0·110	0·145	0·131	0·092	−	−
Beattie	R.F.	0·158	0·142	0·131	0·142	0·124	0·112	−	−
	L.F.	0·140	0·124	0·106	0·131	0·121	0·101	−	−
	R.H.	0·185	0·184	0·167	0·152	0·142	0·116	−	−
	L.H.	0·152	0·142	0·121	0·142	0·121	0·105	−	−
Daisy	R.F.	0·113	0·108	0·089	Not determined			−	−
	L.F.	0·146	0·142	0·121	0·121	0·099	0·077	−	−
	R.H.	0·128	0·113	0·097	Not determined			−	−
	L.H.	0·128	0·110	0·092	,,			−	−
Lily	R.F.	0·156	0·145	0·128	0·156	0·142	0·119	−	−
	L.F.	0·137	0·121	0·092	Not determined			−	−
	R.H.	0·152	0·138	0·119	0·152	0·128	0·109	−	−
	L.H.	0·160	0·149	0·126	0·191	0·156	0·140	−	−
Black Limb	R.F.	0·152	0·138	0·119	0·144	0·121	0·101	−	−
	L.F.	0·128	0·113	0·099	Not determined			−	−
	R.H.	0·129	0·117	0·096	,,			−	−
	L.H.	0·135	0·119	0·099	,,			−	−
Derby	R.F.	0·128	0·121	0·106	,,			−	−
	L.F.	0·167	0·154	0·140	0·135	0·113	0·091	−	−
	R.H.	0·149	0·136	0·126	0·124	0·103	0·085	−	−
	L.H.	0·136	0·121	0·092	Not determined			−	−
Leicester	R.F.	0·319	0·291	0·277	0·328	0·316	0·300		+
	L.F.	0·326	0·308	0·298	0·355	0·326	0·315	Bulk	+
	R.H.	0·140	0·128	0·108	Not determined			+	+
	L.H.	0·144	0·131	0·112	0·149	0·128	0·128		−
Old Melton	R.F.	0·140	0·131	0·124	0·149	0·138	0·124		+
	L.F.	0·340	0·326	0·321	0·333	0·312	0·303	Bulk	+
	R.H.	0·135	0·118	0·108	0·138	0·128	0·110	+	+
	L.H.	0·298	0·282	0·266	0·303	0·277	0·267		+
Gee	R.F.	0·121	0·103	0·085	0·138	0·124	0·099	−	−
	L.F.	0·124	0·113	0·096	0·129	0·118	0·099	−	−
	R.H.	0·124	0·117	0·096	0·149	0·126	0·092	−	−
	L.H.	0·142	0·128	0·121	0·152	0·128	0·105	−	−

* Mastitis streptococci present = + ; mastitis streptococci absent = − .

respectively, than method (*e*). Moreover, the results from samples taken on April 15 are, with a few exceptions, comparable with those obtained on April 3. There are three instances, however, where a wide difference occurred, viz. Barnet (L.F.), Morris (R.F.) and Morris (R.H.). It is probable that the variation in the results of Barnet (L.F.) was due to increased pathological disturbance resulting from infection, but the reason for the exceptional increase in chlorine content of the milk from the R.F. and R.H. "quarters" of Morris is obscure. At the time these samples were taken this cow was in full milk, so that the sudden rise in chlorine content cannot be attributed to advance in lactation.

It has been stated by numerous workers that the chlorine content of normal milk varies between 0·07 and 0·12 per cent. The method of analysis adopted to arrive at these figures has usually been that of determining the chlorine in the ash, consequently, when the chlorine is determined by method (*a*) the limit figures representing variation in normal milk become 0·09–0·15 per cent., *i.e.* 25 per cent. higher. Thus the arbitrary standard of 0·14 per cent. chlorine fixed by Rosell, above which pathological conditions are indicated, falls well within these limits. This undoubtedly accounts for the large number of samples which were indicated as abnormal by method (*a*). By this method, each of the twelve cows whose results are shown in Table III has at least one "quarter" giving milk containing more than 0·14 g. chlorine per 100 ml., whereas ten cows are in this category when using method (*b*) and only six when using method (*e*). As regards the number of "quarters" from which milk with a high chlorine percentage is produced, methods (*a*), (*b*) and (*e*) show 56, 33 and 17 per cent. respectively when the samples from both April 3 and 15 are taken collectively.

It is well established that the chlorine content of milk rapidly increases towards the end of lactation. Sharpe and Struble(9) found that the increase was slight up to 60 per cent. of the lactation period, more rapid from 60 to 90 per cent., after which an abrupt rise occurred. The cows included in Table III were at various stages of lactation, but in no case had more than 70 per cent. of this period been covered. Thus it is unlikely that the chlorine results obtained were influenced by the stage of lactation.

The results of the cultural examination as shown in Table III were obtained from samples taken on March 7 and April 15. On March 7 bulk samples only were taken from Barnet, Leicester and Old Melton, but at the repeat test on April 15 "quarter" samples were examined. It will be observed that there was no variation in the bacteriological results obtained from "quarter" samples taken on these two dates. From the positive "quarters" recorded in Table III mastitis streptococci were isolated, culturally similar to the Haemolytic Group I of Minett *et al.*(16).

Towards the end of March 1935 the infected animals were segregated and milked last. All fresh animals were tested before being added to the herd. Following the segregation, "quarter" samples from the "clean" portion of

the herd were examined bacteriologically at frequent intervals. The fourteen cows which were found negative in March 1935 have remained so during the 10 months these examinations have been continued. Furthermore, no clinical cases of mastitis have been observed in the "clean" portion of the herd during this period.

When the chlorine and bacteriological results are compared, it will be observed that of the fifteen "quarters" positive culturally, eight had a chlorine content not greater than 0·14 per cent. by method (*a*), nine by method (*b*) and eleven by method (*c*). That samples of infected milk are encountered where the chlorine content falls within the limits of normal milk has been observed by Leitch(17), Sharpe and Struble(9) and others. Of the remaining thirty-three "quarters", negative culturally, nineteen had a chlorine content greater than 0·14 per cent. by method (*a*), ten by method (*b*) and four by method (*c*).

The large number of "quarters" yielding milk with a high chlorine content by method (*a*) is not surprising when it is considered that this method gives results which are approximately 25 per cent. in excess of those obtained by method (*c*).

In the case of Wissendine it is of special note that the L.H. "quarter" yielded milk which had a low chlorine content but was positive culturally, whereas the L.F. "quarter" yielded milk which reacted *vice versa* to both tests. This anomaly was confirmed by taking further samples. Some 6 weeks later the L.H. "quarter" showed marked clinical symptoms of mastitis.

Summary

1. The importance of a rapid and simple test for the diagnosis of mastitis in cows is a question which has attracted considerable attention on the part of many workers. One such test based on the determination of chlorine in milk by a rapid method has been advocated recently by Rosell.

2. The authors of this paper have made a careful examination of this method and compared the results obtained on samples of milk with those given by other methods known to yield reliable and reasonably accurate results.

3. The direct titration of chlorine in milk with silver nitrate solution, without the removal of proteins, invariably gives high results, and, moreover, a satisfactory end-point is difficult to obtain.

4. Rosell's claim that "quarter" samples of milk giving a chlorine figure in excess of 0·14 per cent. indicate the presence of mastitis does not appear to be justified in view of the results obtained by the authors for various samples of milk from cows known to be infected with, or free from, mastitis streptococci.

5. The chlorine figures as determined by reputable methods, which were obtained from the milk of cows in a herd where mastitis was rampant, have been compared with the results of bacteriological examination. From these

comparisons it is evident that samples of milk with a chlorine content within the range of normal milk, *i.e.* 0·07–0·12 per cent., may be derived from "quarters" infected with mastitis streptococci whilst, on the other hand, cows may yield milk with a chlorine content well above these limits and yet no evidence of the presence of mastitis is forthcoming.

6. From these experiments it is claimed that the determination of the chlorine content of milk is not a reliable method for the ascertainment of the presence or absence of mastitis in dairy cows.

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130. BACTERIOPHAGE PHENOMENA IN CULTURES OF LACTIC STREPTOCOCCI

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(With 1 Plate and 2 Figures)

In a previous paper(1) the possible causes of loss of activity by cheese "starter" cultures were discussed. It was shown that aeration of a milk medium in which lactic streptococci were to be grown sometimes led to a sudden failure in growth, and the suggestion was made that the root cause was a physiological peculiarity in the bacteria. Later work has shown that it is unnecessary to postulate such a peculiarity, since failures of the type previously described are due to the action of bacteriophage on the streptococci.

A note(2) recording the observation of the occurrence of bacteriophage in starter cultures has already been published; the object of the present paper is to describe in detail the properties and mode of action of the phages which act upon lactic streptococci.

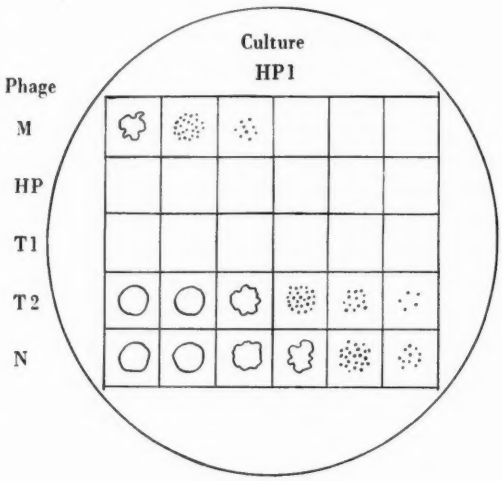
Demonstration of the presence of bacteriophage in a starter culture

The presence of phage in a starter culture was first recognised during a renewed investigation of the phenomenon of failure in activity caused by aeration of the milk. A pure culture of a strain of *Str. Cremoris*, which was in commercial use as a cheese starter, suddenly become erratic in its behaviour from day to day. The mother culture appeared quite normal, and it was possible to demonstrate that of two batches of starter made side by side, one which had been stirred vigorously at the time of inoculation, was more liable to lose its activity. In this particular instance loss of activity was not manifested by complete failure of growth in the first generation. The batch of starter prepared in stirred or aerated milk was quite normal in appearance and acidity; it failed, however, to produce acid when added to milk in the cheese vat.

In an attempt to detect the essential difference between the streptococci in the active and inactive batches of starter culture, a few drops of each were added to tubes of pasteurised milk coloured with methylene blue and held at 30° C. The blue in both tubes was decolorised in approximately equal times (2–3 hours), and smears made from the tubes at that time showed under the microscope a copious growth of normal streptococci in both. An hour later it was observed that the blue colour had returned to the tube to which the

inactive starter had been added, and a smear examined under the microscope showed a total absence of bacteria. In the case of the active starter, growth progressed normally until the milk finally clotted.

It was evident that some agent had caused a lysis of the streptococci in the inactive culture and that the lysis in turn accounted adequately for a failure in acid production in the cheese vat. The rapidity with which the organisms disappeared suggested the action of a bacteriophage, and an attempt was therefore made to demonstrate the presence of such an agent. It was found that minute quantities of the milk from the tube in which the strepto-



Text-fig. 1.

cocci had undergone lysis sufficed to restart the sequence of events in an active young culture of the susceptible strain. The whole process could then be carried on from tube to tube, proving that the phenomenon was one of transmissible lysis.

The final link with phages described by other observers was provided by the demonstration of "plaques" on a solid medium. A clotted starter culture which was known to be inactive as a result of aeration was spread thickly on the surface of a plate of yeast whey agar. After incubation at 30° C. overnight the dense, almost confluent, growth was dotted with clear circular "plaques" which corresponded with the classical descriptions of bacteriophage (see Plate I).

When the same inactive culture was streaked sparingly over the surface of yeast whey agar so that discrete colonies were produced after incubation at 30° C., a proportion of the colonies were observed to become gradually transparent until only their "ghosts" remained. The phage could be propagated from these transparent colonies.

Purification and preparation of suspensions of phage

After several trials, the following method for the preparation of a pure suspension of the phage was developed. One of the plaques on the first plate culture was touched with a sterile wire, and the trace of phage so obtained was transferred to a tube of sterile milk. The milk was then inoculated with a drop of the susceptible strain of streptococcus and phaging was allowed to occur at 30° C., the process being followed by microscopic examination from time to time; lysis was usually complete in from 8 to 12 hours. A larger bulk of sterile milk (250–300 ml.) was then inoculated with the streptococcus, and a drop of the phaged culture was added.

Phaging in this second generation was usually complete in 4 or 5 hours at 30° C. As soon as lysis had taken place rennet and calcium chloride were added to the culture, and after a firm clot had formed as much whey as possible was expressed. The whey was filtered through linen and then through a sterilised Seitz filter. This yielded a clear fluid free from bacteria and containing a high concentration of phage.

Properties of the streptococcal phage

Most of the properties of the first phage prepared corresponded in a general way with those described for phages for other groups of bacteria. The most striking feature was its great activity. Observations on streptococcal phages in the literature are somewhat scarce in any case, and many of those reported have been so weak that they were lost after one or two passages (3, 4). In some instances (5) the presence of phage in streptococcal cultures has merely been inferred from variations in the appearance of colonies on agar. The first phage isolated during the course of the present work showed an activity comparable with that of the most powerful phages described for staphylococci and Gram-negative bacilli. Various preparations gave plaque counts of the order of hundreds of thousands of millions per millilitre.

During most of the work estimations of activity were made by the somewhat rougher method of serial dilution. Loopsfull of various dilutions of the phage in sterile water were spread on marked areas of a plate of yeast whey agar (2 per cent. agar) on which had previously been spread a culture of the susceptible strain of streptococcus. The loop used was found to hold on the average a drop amounting to 1/500 ml. The dilution at which evidences of phage action just failed was taken as the titre of the phage. By this method titres of 10^{-8} and 10^{-9} were commonly obtained. The lower dilutions showed circles of confluent lysis, and the higher dilutions showed isolated plaques which became progressively fewer with rise in dilution.

The plaques were much smaller than those described for phages against the Gram-negative bacilli; their average diameter was 0·5 mm., with variations depending on the thickness of the bacterial growth.

The optimum temperature of the phage was about 30° C.; it acted on the streptococci at 20 and at 37° C., although more slowly at both these temperatures. This has a bearing on the repercussions of phage action in cheese manufacture. A starter culture is usually incubated at 20° C.; in the cheese vat it is maintained at temperatures varying from 30 to 37° C. It is quite evident from our experience that a phage in a starter culture may give no visual evidence of its presence until the starter is mixed with milk in the cheese vat at 30° C. The influence of temperature is, however, by no means so important as the influence of aeration of the medium. The phenomenon, as we originally observed it, was a "flare up" of phage caused by aeration of the milk. This action took place at 20° C. There is therefore some factor other than temperature which normally holds the phage in a dormant state in the culture.

The action of the phage could be demonstrated in media other than milk. In whey broth at 30° C. a clearing of the turbid culture could usually be observed after 5 or 6 hours; several potent suspensions of phage were prepared in this way. Milk, however, gave by far the most consistent results as a medium, as would be expected from the fact that no medium yet discovered compares with milk for the growth of lactic streptococci. Most observers agree that phage multiplies most readily in the medium which best suits the susceptible organism.

In the matter of specificity, the streptococcal phage seemed to differ from most of the other phages previously reported. The first phage isolated in the course of this work was tested against several stock strains of lactic streptococci, but it proved active only against the particular strain of *Str. Cremoris* (R 3) from which it had been isolated; this specificity has been maintained. No deliberate attempt has, however, been made up to the present to adapt it to other strains. This specificity of phage to one strain might perhaps be expected in view of the antigenic nature of the streptococci, since Burnet in several papers has shown the close relationship between antigenic structure and sensitivity to phage. In a recent paper(6), Burnet and Lush, however, do not find the staphylococcal phages similarly specific.

The phage preparations obtained by rennet action on milk were quite stable. They were normally kept at 4° C., and suffered no significant loss in power over a period of several months. A small amount kept at room temperature during fairly warm weather for over a week showed only slight loss in power. The phage was almost completely destroyed by exposure to 70° C. for 30 min.

Origin of the phage

There are some workers who believe that phage can be produced on occasion by normal cultures. Others maintain that phage is never produced spontaneously, but arises from contamination from outside sources; and the apparently spontaneous appearances which occur with pure cultures are considered to be due to some change in the conditions of culture which favours

the development of the phage. Burnet (7) attempts to reconcile the two points of view in a rather ingenious way. He suggests that there may be an analogy between phage and the malignant cell in mammalian cancer.

The malignant cell originates as a variation of the normal cell. When transplanted from host to host, it lives and develops as an independent parasite. However, as Burnet points out, there is insufficient evidence to settle the question at present, since it is impossible with our present methods to decide whether or not a pure culture is infected with a phage even when the latter gives no sign of its presence.

In the present instance there are some peculiar features which do not seem to have been observed in connection with phage action on other types of bacteria, and which may have some bearing on the nature and origin of phage. The effect of aeration of the milk medium was outstanding. The main features of this have already been described, since it formed the starting-point of these investigations; but its implications became fully realised only after a more detailed examination of phage action. A "sensitive" culture of lactic streptococci grown in non-aerated milk gave in general no indication of the presence of even a trace of phage. A clear filtrate from a 24-hour culture had no effect on the parent strain, either on the surface of agar or in a liquid medium. Out of six similar cultures in aerated milk, however, two or three would show the presence of a high concentration of phage which had "flared up" overnight, in some cases to an extent sufficient to prevent the clotting of the milk.

Repeated purification of the culture did not eliminate the phenomenon. One culture was replated eight times and yet gave a phage when grown in aerated milk. d'Herelle (8) and Schwartzman (9) found that aeration increased the activity of phage, but the differences observed were merely quantitative and relatively small. Aeration in our experiments gave an almost qualitative difference which might be taken at first sight as evidence of the production of phage from a normal bacterium. Otherwise it must be assumed that phage multiplied to an infinitesimal degree with the culture, and clung to every colony on a plate culture. With some cultures which were extremely "sensitive" to aeration, however, it was possible to demonstrate the presence of phage simply by incubation at 30° C. instead of the usual 20° C., when self-phaging of the culture occurred in 5–6 hours.

The variations in "sensitivity" to aeration which were reported in a previous paper (1) indicate that phage must wax and wane to some extent in a culture subcultured daily and kept at 20° C. Most of the time the phage must be present in infinitesimal amounts and it seems, moreover, to exist in a peculiar state of relationship to the bacteria; the term "occluded" might perhaps be used. Aeration of the milk seems in certain cases to be a "trigger" which liberates the phage and enables it to multiply in a more normal manner.

A further point in connection with aeration is the composition of the milk medium. Milk from different sources seems to vary in its effect upon a culture which contains an "occluded" phage. This has been observed mainly in work

in the field, and although absolute control of all the factors is difficult under such circumstances, it has been observed sufficiently often to render it fairly certain that there is an actual difference. No attempt has been made up to the present to correlate this variation in the ease with which a phage "flares up", with the chemical composition of the milk. It seems hardly likely that quantitative differences in the main constituents could have such an effect; the key point must lie in the quantitative difference in some minor constituent, or a qualitative difference in a major constituent. In this connection, the finding recorded previously(1), that yeast extract had an influence under certain conditions, has a bearing.

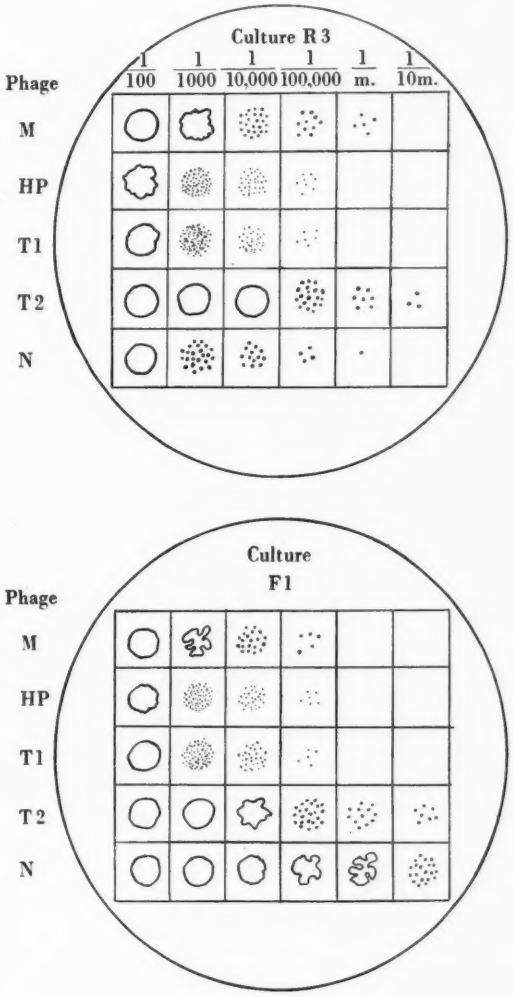
Attempts to produce immune forms

From a practical point of view the greatest interest is attached to possible means of preventing the action of bacteriophage. Soon after the isolation of the first phage, attention was paid to the possibility of developing an immune strain. The susceptible strain of *Str. Cremoris* (R 3) was inoculated into sterile milk containing a trace of the phage (M). The process of phaging was complete in about 5 hours at 30° C. The milk sample, apparently free from all bacteria, was kept in the incubator at 30° C. until, in 2 days' time, a secondary culture had developed sufficiently well to cause clotting of the milk. When this culture was plated on yeast whey agar, and several colonies were picked into sterile milk, a few of the resulting cultures were found to produce acid just as actively as the original strain (R 3). One of these cultures (F 1) proved to be quite efficient as a starter in the cheese vat. It was relatively resistant to the action of phage M; high concentrations of the phage would attack it, especially on solid media, but low concentration showed no evidence of action. For instance, a certain phage preparation had a titre of 10^{-6} on culture R 3; its titre on F 1 was only 10^{-2}. In other respects F 1 was indistinguishable from R 3. Culture F 1 was sent to several commercial cheese factories, and in some of these it gave no trouble. In others, however, it failed suddenly just as R 3 had done, with all the appearance of phage action. Samples examined showed the presence of a phage which had evidently, from a description of the circumstances, "flared up" suddenly, as a result of aeration (which can hardly be avoided under the conditions obtaining in some factories).

The new phage (HP) was, however, quite different in action to phage M. It attacked cultures R 3 and F 1 to an equal extent, it showed smaller plaques on solid media, and had a lower thermal death-point (50° C. as compared with 70° C.). The process of immunisation was repeated and a third strain (HP 1) was prepared. The same series of events repeated themselves, and it soon became evident that this process of immunisation was not a permanent solution for the trouble. In addition to the appearance of more phages, the immune (?) strains gradually lost their resistance to the phage with which they had been treated during the course of their isolation. Text-fig. 2 shows in diagrammatic form the results of an experiment in which the action of five

phage preparations was tested against three cultures, all of which had originated from culture R 3.

The cultures were therefore identical except for their reactions to the phages. The rings indicate confluent lysis of the bacterial growth over the



Text-fig. 2.

area where the loopful of phage dilution was spread; the dotted areas are a representation of isolated plaques, the numbers and sizes of dots giving some indication of the numbers and sizes of the plaques.

At the time of the experiment culture F 1 had nearly lost its resistance

to phage M. Culture HP 1 was still resistant to phages HP and T 1. It is interesting to note the effects of phages T 2 and N on cultures R 3 and F 1.

It was considered for some time that the successive phages were the result of contamination under commercial conditions, but eventually it became evident that the immunised strains even under controlled conditions in the laboratory would yield phages which "flared up" when the milk medium was aerated. The phages appeared more readily in some batches of milk than in others. This successive appearance of what are apparently different phages on one strain of bacterium raises further interesting points on the nature of phage. More information is necessary before any hypothesis can be developed.

SUMMARY

The occurrence and isolation of bacteriophages from cultures of lactic streptococci ("starter cultures") is described. Aeration of the milk in which the phage-infected cultures are grown is shown to have a stimulating action on the development of the phage. The characteristics of phages for lactic streptococci are described.

The authors' thanks are due to Prof. W. Riddet, and Dr F. H. McDowall for their constant interest and constructive criticism.

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Plate culture of *Str. Cremoris* showing phage plaques
(about 1½ times normal size).

637.127:576.8:637.333.9

131. A STUDY OF THE BACTERIAL FLORA OF FOREMILK AND OF RENNET EXTRACT, WITH SPECIAL REFERENCE TO ACID-PROTEOLYTIC TYPES

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Previous investigators have shown that organisms producing both acid and proteolysis simultaneously can be isolated from the majority of milks and milk products. Attention has been drawn to the presence of such types in the udder of the cow by Gorini(1), and many subsequent workers(2,3,4,5,6,7). Orla Jensen(8) has noted their presence in market milk, and they were found in several types of cheese by Freudenreich(9, 10), Orla Jensen(8, 11) and others(12, 13). Gorini(14, 15, 16, 17) has especially ascribed to this group of bacteria an important function in the ripening of various cheeses, asserting that proteolysis in an acid medium was an essential characteristic of cheese-ripening organisms. Present-day knowledge shows that acid-peptonising strains are undoubtedly present in milk, and that some species such as the lactobacilli play an important part in producing flavour in ripening cheese.

The present investigation was carried out mainly to study the acid-proteolytic flora of foremilk and rennet extract. The significance of these types in the ripening of Cheddar cheese will be dealt with in a subsequent communication.

Investigations on the foremilk

Experimental

The investigation was carried out on samples of foremilk taken from four healthy cows selected from the herd of the National Institute for Research in Dairying, Shinfield. Samples were collected into sterile flasks under aseptic conditions and were plated out directly in dilutions up to 1 in 100 on the following media:

Litmus lactose gelatin: incubated at 22° C.

Litmus lactose agar: incubated at 30° C.

Nutrient agar: incubated at 30° C.

A count was made after 40 hours' incubation (Table I) to show individual sample differences, and representatives of all colonies were subcultured at frequent stages over a period of 6 weeks.

Table I. *Counts per ml. of foremilk after* 40 *hours' incubation*

Medium	Cow 1	Cow 2	Cow 3	Cow 4
Nutrient agar	60	70	2560	150
Litmus lactose agar	40	65	3220	145
Litmus lactose gelatin, number of liquefiers	0	7	1200	6

Of sixty-three cultures obtained from the plates, fifty-eight proved to be cocci, four were diphtheroids and one was an actinomycete. The following technique was adopted for the purposes of identifying and classifying the cultures:

Action in litmus milk. The times of acid development, coagulation and peptonisation were especially noted.

Action in chalk litmus milk. Was found to be of greatest use in demonstrating the presence or absence of bacterial rennin. Litmus milk plus 5 per cent. of precipitated chalk was very suitable if the cultures were shaken every 12 hours.

Liquefaction of gelatin. Lemco gelatin at *p*H 7·6 was used and the time and character of liquefaction noted in stab cultures incubated at 20° C.

Proteolysis. Was primarily observed macroscopically in litmus milk cultures. In a subsequent paper figures will be given for (*a*) the increase in water-soluble nitrogen and (*b*) the increase in amino nitrogen resulting from inoculation of the cultures into sterile milk.

Carbohydrate fermentation. Acid production in the various sugar broths was stimulated by the addition of 0·2 per cent. marmite. Marmite was also incorporated in the nutrient agar slopes used for preserving the cultures, with satisfactory results. None of the isolated organisms produced gas from the eight test sugars used, dextrose, lactose, sucrose, maltose, mannitol, inulin, salicin, raffinose.

Reduction of nitrates. Nitrites were tested for at intervals of 4 days.

Indol formation. One ml. of the broth culture was tested daily with Ehrlich's reagent.

Haemolysis. Was detected by streaking 24-hour growths of the organisms on blood agar plates (nutrient agar plus 10 per cent. citrated cow's blood). The plates were incubated at 37° C. for 3 days and readings taken every 24 hours.

Pigment formation. Was observed on potato slopes immediately subsequent to isolation in order that its intensity should not be decreased by artificial culture.

Classification of the isolated cultures

The fifty-eight cultures of cocci fell into three main groups, *i.e. Staphylococcus*, *Micrococcus* and *Streptococcus*. The power to haemolyse blood readily separated the staphylococci from the micrococci, but the same did not hold good for the *Streptococcus* group, since haemolytic members were also present. In this instance it was possible to differentiate the streptococci from the micrococci and the staphylococci solely on the basis of morphological and biochemical characteristics. In presenting this classification, names have been tentatively used for cultures whenever close agreement was shown to cultures listed in Bergey's *Manual of Determinative Bacteriology*. A brief note on the groups is given below, a more detailed summary of biochemical characters appearing in Table II.

Table II. *Group characteristics of the foremilk cocci*

Group	No. of cultures	Carbohydrate fermentations								Nitrate reduction	Gelatin		Litmus milk			Haemolysis	Pigment	Species
		Dext.	Sucr.	Lact.	Malt.	Mann.	Inul.	Salic.	Raff.		Liquefaction	Form	Acid	Coag.	Pept.			
I	11	+	+	+	+	+	−	−	−	+	+	Saccate	+	+	−	+	Orange	*Staphylococcus aureus* (Rosenbach)
I*a*	2	+	+	+	+	.	−	−	−	+	+	Saccate	+	+	−	+	Orange	*Staphylococcus* sp.
II	5	+	+	+	+	+	−	−	−	+	+	Infundibuliform	+	+	−	−	Fawn	Not named
II*a*	2	+	+	+	+	−	−	−	−	+	+	Saccate	+	+	−	−	Orange	Not named
III	13	+	+	+	+	−	−	−	−	−	+	Variable	+	+	−	−	Yellow	Not named
III*a*	2	+	+	+	+	+	−	−	−	−	+	Variable	+	+	−	−	Yellow	Not named
IV	2	+	+	+	+	+	−	−	−	+	+	Crateriform	+	+	+	−	Lemon-yellow	*Micrococcus caseolyticus* (Evans)
V	1	+	+	+	+	−	−	−	−	−	+	Infundibuliform	+	+	+	−	Lemon-yellow	*Micrococcus freudenreichii* (Guillebeau)
VI	2	−	−	−	−	−	−	−	−	−	+	Stratiform	−	−	−	−	Yellow	*Micrococcus flavescens* (Henrici)
VII	3	+	+	+	+	−	−	−	−	+	−	−	+	−	−	−	Golden-yellow	*Micrococcus varians* (Migula)
VIII	4	+	+	+	+	−	−	+	−	−	−	−	+	−	−	+	White	*Streptococcus pyogenes* (Rosenbach)
IX	8	+	+	+	+	+	−	+	−	−	+	Saccate	+	+	+	−	White	*Streptococcus liquefaciens* (Orla Jensen)

Table III. *Numbers of micro-organisms per ml. of rennet extract*

Sample	Litmus-lactose agar			Dextrose-bean agar			Casein agar		
	37°C.	30°C.	20°C.	37°C.	30°C.	20°C.	37°C.	30°C.	20°C.
I	694	2,264	6,100	185	1,267	9,300	145	1,040	10,800
II	17,600	27,900	31,800	13,300	29,500	19,100	16,100	24,300	27,000
III	7,600	25,600	32,900	2,500	15,600	55,000	12,100	69,600	95,000

Staphylococcus *group.*

The thirteen cultures classified in the *Staphylococcus* group were haemolytic and biochemically active. Growth on artificial media was luxuriant, and an oily orange streak was obtained on potato. Eleven of the cultures gave a strong acid coagulation in litmus milk and were typical strains of *Staphylococcus aureus* (Rosenbach). The remaining two cultures were actively acid-proteolytic in milk. One of these strains failed to ferment mannitol. All thirteen cultures were characterised by producing a high hydrogen-ion concentration in glucose phosphate broth.

Micrococcus *group.*

The thirty cultures classified in this group were non-haemolytic and varied considerably in their biochemical activities. Six subgroups were differentiated and are fully considered in Table II. Group IV containing two cultures was biochemically similar to the type *Micrococcus caseolyticus* described by Evans(7), except that the rapid and complete peptonisation claimed was not so evident. Proteolysis in the above two cultures tended to become less vigorous as the period of artificial culture increased. Proteolysis in both cases was preceded by acid formation demonstrating the acid-proteolytic nature of the group. Digestion of the casein in chalk litmus milk was of a more complete nature. Group V containing one culture was distinguished from group IV by its inability to reduce nitrates to nitrites and to ferment mannitol. The organism in culture was decidedly slimy and when initially isolated was so strongly acid-proteolytic that only a small plug of undigested casein remained at the base of the inoculated milk tube. The characters of the group appear to be identical with the listed description of *Micrococcus freudenreichii* (Guillebeau).

Streptococcus *group.*

Twelve cultures were classified in the *Streptococcus* group. Of these, four produced green-zoning (alpha-haemolysis) on blood agar plates, readily fermented salicin but failed to ferment mannitol. The growth of these cultures on nutrient agar was sparse and discrete; in litmus milk acid was formed slowly. These four alpha-haemolytic streptococci appear to be allied to the pyogenic streptococci. The remaining eight cultures were typical vigorous strains of *Streptococcus liquefaciens*, non-haemolytic, characterised by a scanty white growth on artificial media, the fermentation of salicin and mannitol and a strong acid peptonisation of litmus milk. Coagulation in chalk litmus milk was complete in 12–15 hours, and proteolysis was more vigorous and complete under these alkaline conditions.

The remaining three cultures isolated from the foremilk were pink cocci belonging to the genus *Rhodococcus*.

Discussion

The most noticeable feature of the above investigation was the relatively small number of acid-proteolytic cocci in the foremilk. From the fifty-eight cultures of cocci isolated only four types were capable of digesting casein under acid conditions brought about by their own activities, namely: *Staphylococcus* sp., *S. liquefaciens*, *Micrococcus caseolyticus*, *M. freudenreichii*. Of these the only cultures which were vigorously acid-proteolytic were the strains of *Staphylococcus* and *Streptococcus liquefaciens*. The other acid-peptonising strains *Micrococcus caseolyticus* and *M. freudenreichii* did not produce the vigorous casein degradation claimed by some investigators for these organisms.

Neglecting for a moment the presence of *Streptococcus liquefaciens*, out of the remaining forty-eight cultures only five, or 10·4 per cent. of the total, were acid-proteolytic in milk. On the other hand, forty-eight out of fifty-five cultures, or 87·3 per cent., were capable of liquefying gelatin, but at the same time it is realised that the possession of a gelatinase is not necessarily indicative of true proteolysis. Accordingly the figure of 87·3 cannot be regarded as indicating true peptonising strains, the exception being that these forms may be capable of decomposing the simpler forms of nitrogen compounds found in ripening cheese. It seems, therefore, that a small number of acid-proteolytic cocci are transmitted *via* the udder to milk, and by reason of their own constitution these types are capable of resisting the subsequent changes due to acid development. Consequently they may produce their own special reactions in milk and milk products where other types would be inhibited by the deleterious acid formation of the vigorous lactic acid streptococci.

The occurrence of *Streptococcus liquefaciens* in the udder of the healthy cow has been recorded previously by Burri and Hohl(18). Its presence in the udder is a potential danger during summer months, inasmuch as under suitable conditions sweet curdling of the milk may occur. In cheese ripening the position and importance of *S. liquefaciens* is somewhat variable, depending largely on the type of cheese made. According to Orla Jensen(19) the liquefying cocci are important in producing the characteristic taste of Tilsiter cheese, and may aid in the ripening of Gouda cheese and other non-acid varieties. In English Cheddar *S. liquefaciens* is renowned for its deleterious action in producing a bitter flavour. With an abundant infection, the cheeses may be quickly reduced to a mass of partially digested liquefying casein.

As was found in this investigation, *Staphylococci* are commonly isolated from the udder. It is probable that the percentage of these types in the udder increases with local inflammatory infections. The presence of the yellow liquefying cocci in the udder does not appear to possess any significance other than that such types may aid in the final breakdown of split protein products in cheese. Their constant presence in the foremilk leads one to suspect that contamination from the air *via* the teat canal is their most probable source.

Investigations on rennet extract

Experimental

Three samples of rennet extract were investigated as follows:

Sample I: approximately 3–4 months old.

Sample II: 2 weeks old.

Sample III: probable age 3–6 months, and of a different manufacture from samples I and II.

These samples were plated out in triplicate on litmus-lactose-peptone agar, dextrose-bean extract agar and casein-digest agar, and the plates incubated at 20, 30 and 37° C. for 10 days in each case in order to compare the counts at different temperatures. Table III (p. 65) shows the results.

The differences noted in the general appearance of the three sets of plates were so striking that they deserve mention.

Description of the plates of sample I

The higher temperatures quickly stimulated the growth of spore formers, yeasts, moulds and actinomyces, the lower dilution plates being useless for isolation purposes. At lower temperatures the plates were clearer at first, and the flora was found to consist largely of alkali-forming yeasts. A variety of colonies appeared on the litmus-lactose agar plates at 30° C., many being pigmented acid-forming cocci. In addition there were present on all the plates large numbers of small lenticular subsurface colonies shown later to be Gram-positive short rods. A differential count was made on the litmus-lactose agar plates at 30° C. of acid and alkali-forming types, namely: acid-forming 642 per ml., alkali-forming 120 per ml. The remaining types were the lenticular subsurface colonies producing no colour change in the litmus.

Description of the plates of sample II

The plates at all temperatures were particularly free from yeasts, moulds and actinomyces. A few spore-formers occurred spasmodically on the lower dilutions, and in a lesser degree the same was true of the yeast colonies. The majority of developing types were acid-forming orange chromogens, and, excepting a yellow colony appearing on the one-tenth dilution of the plates, these orange types were present in pure culture. They were subsequently shown to be cocci, similar to one another in morphology and staining reactions. As compared with the plates of sample I, two principal features were evident:

(1) The absence of moulds, yeasts and actinomyces in the fresh sample of rennet.

(2) The uniformity of the flora in the fresh rennet sample and its restriction to practically one type—the orange chromogens.

Description of the plates of sample III

Although possessing a higher count than the two previous samples, sample III did not exhibit the variety of types shown by those samples, and acid-forming colonies were not so prevalent. A noticeable feature was the extensive contamination of the sample with yeasts, moulds and actinomyces. The moulds were green Penicillia. Slight alkali-forming yeasts were especially numerous at the lower incubation temperatures and spore-formers at the higher ones. The number of actinomyces was perhaps surprisingly high, a count of 400 per ml. being recorded on litmus-lactose agar incubated at 30° C. Micrococci were but sparsely represented on the media used at each of the three incubation temperatures, the majority of such types being acid-forming orange chromogens similar to those found in the fresh sample of rennet.

The higher counts of sample III were accounted for by the development of numerous subsurface colonies. They first appeared on casein agar incubated at 30 and 37° C. after 4 days, at later dates they appeared on the other media and also on the plates incubated at 20° C. Microscopical examination showed these types to be Gram-positive cocci occurring in pairs and short chains. The cultures were considered to be streptococci and have been detailed in Table VI.

It is reasonable to suppose that the fresher the sample of rennet the greater is the possibility of that sample containing bacterial types common to rennet extract. In the case of sample II, which was only 2 weeks old, the flora was composed chiefly of acid-forming orange cocci, and these types would appear to constitute the normal fresh rennet flora. It was noteworthy that similar types occurred in both the older rennet samples examined. The presence of yeasts, moulds, actinomyces and spore-formers in the older samples of rennet appeared to indicate casual contamination, since in the fresh sample such types were almost absent. Yeasts were found in all samples, but only sparingly in sample II, and it is probable that they may in part have been derived from the original "vell".

Classification of the organisms isolated from rennet

The examination and classification of the organisms isolated from rennet were conducted on similar lines to that adopted for the udder cocci. No attempt was made to classify the yeasts, moulds, actinomyces and spore-formers encountered apart from the following general characters. Spore-formers were characterised by an alkaline peptonisation in milk, rapid reduction of nitrates to nitrites and an acid fermentation in sucrose broth with no gas evolution. The yeasts present were mainly inactive in milk, but one species was isolated that liquefied gelatin and rapidly peptonised milk. The actinomyces were chalky white species, liquefying gelatin and being actively proteolytic in milk. The Gram-positive short rods were inactive in milk but liquefied gelatin slowly.

The cocci were investigated in detail. It was found that individual differences in like strains made the classification of the rennet cocci somewhat complex, it being difficult to decide how far these differences should allow of a separate grouping of the cultures. As with the foremilk, haemolysis was considered the primary differential test to distinguish the staphylococci from the micrococci, and consequently several haemolytic strains had to be included in the *Staphylococcus* group that were not in agreement with listed cultures in Bergey's *Manual of Determinative Bacteriology*. To simplify the classification it was decided to present the various groups and subgroups in the form of tables under the headings *Staphylococcus*, *Micrococcus* and *Streptococcus*. This has been done in Tables IV, V and VI, where the characters necessary for the classification of the cultures are set forth in detail.

Discussion

The investigation proved rennet extract to be a more fruitful source of proteolytic cocci than the foremilk. Among the haemolytic cocci a variety of acid-proteolytic strains were encountered, both orange and yellow types. In addition, cultures were isolated that failed to produce acid in milk, but were otherwise similar to the proteolytic acid-forming haemolytic strains. Of the micrococci, group V, represented by two cultures similar to *Micrococcus caseolyticus* (Evans), was strongly acid-proteolytic, this activity being maintained throughout a long period of artificial culture. The cultures were isolated from different rennet samples and possibly *M. caseolyticus* may be present in small numbers in the majority of rennet extracts. Group XV, also classified in the micrococci, was strongly proteolytic in milk but failed to produce acid. Cultures were ropy and the strain appeared to be allied to the type *Micrococcus freudenreichii* (Guillebeau)—possibly a variant strain. Of the streptococci only one group, group XVIII, was acid-proteolytic, the one included culture being identified as *Streptococcus liquefaciens* (Orla Jensen).

In all, sixty-four cultures of cocci were obtained from the three rennet samples, and of these ten cultures or 16 per cent. were shown to be proteolytic in milk and seven cultures or 11 per cent. acid-proteolytic. In older samples of rennet proteolytic cocci were found to be more frequent than in the fresh sample where probably their presence was obscured by the preponderance of orange chromogens. Thus, if the orange cocci are excluded, the above figures assume more importance, namely, that out of forty-four cultures obtained from the older rennet samples eight cultures or 18·2 per cent. were proteolytic and seven cultures or 15·9 per cent. acid-proteolytic when inoculated into milk. These figures and results show that rennet extract may contain a varied flora of proteolytic cocci that under normal cheese-making conditions would gain entrance to the cheese.

A noticeable feature of the investigation was the restriction of bacterial types in the fresh rennet, as compared with the varied bacterial flora occurring

Table IV. *Classification of the staphylococci (haemolytic cocci) isolated from rennet extract*

	No. of cultures	Carbohydrate fermentation								Nitrate reduction	Pigment	Gelatin liquefaction		Litmus milk		
		Dext.	Sucr.	Lact.	Malt.	Mann.	Inul.	Salic.	Raff.			Time days	Form	Acid	Coag.	Pept.
Group I, orange:																
Type I	1	+	+	+	+	+	−	−	−	+	Orange	10	Crateriform	+	+	−
,, II	4	+	+	+	+	+	−	−	−	+	Orange	15	Crateriform	+	−	−
,, III	2	+	+	+	+	+	−	−	−	−	Orange	7	Crateriform	+	−	−
,, IV	11	+	+	+	+	+	−	+	−	+	Orange	3	Crateriform	+	−	−
,, V	2	+	+	+	+	+	−	−	+	−	Orange	10	Crateriform	+	−	−
,, VI	1	+	+	+	+	+	−	−	−	−	Fawn	.	.	+	−	−
,, VII	1	+	+	+	+	−	−	−	−	+	Dull orange	5	Saccate	+	+	+
,, VIII	1	+	+	+	+	−	−	−	−	−	Orange	5	Infundibuliform	+	+	+
,, IX	1	−	−	−	−	−	−	−	−	−	Fawn	.	.	−	+	+
,, X	3	+	+	+	+	−	−	+	−	−	Orange	3	Saccate	+	−	−
Group II, yellow:																
Type I	1	+	+	+	+	+	−	−	−	−	Pale yellow	40	Crateriform	+	+	−
,, II	1	+	−	+	+	−	−	−	−	+	Pale yellow	12	Stratiform	+	+	−
,, III	1	+	+	+	+	−	+	+	−	−	Pale yellow	8	Stratiform	Alk.	+	+
,, IV	1	+	−	+	−	−	−	−	−	−	Yellow	7	Stratiform	+	+	+
,, V	1	+	−	−	−	−	−	−	−	−	Yellow	3	Infundibuliform	+	+	+
Group III:																
Type I	2	+	+	+	+	−	−	−	−	+	Creamy white	.	.	+	−	−

Table V. *Classification of the micrococci isolated from rennet extract*

Group	No. of cultures	Carbohydrate fermentation								Nitrate reduction	Pigment	Gelatin liquefaction		Litmus milk		
		Dext.	Sucr.	Lact.	Malt.	Mann.	Inul.	Salic.	Raff.			Time days	Form	Acid.	Coag.	Pept.
IV	7	−	−	−	−	−	−	−	−	−	White	.	.	−	−	−
V	2	+	+	+	+	−	−	−	−	+	White	4	Crateriform	+	+	+
VI	2	+	−	−	+	−	−	−	−	−	Pale yellow	3	Infundibuliform	Alk.	−	−
VII	2	+	− +	−	−	−	−	−	−	+	Pale yellow	.	.	− Alk.	−	−
VIII	2	+	+	+	+	+	−	+	+ −	−	Yellow	5 12	Stratiform Stratiform	+	−	−
IX	1	+	+	+	+	+	−	−	−	+	Yellow	.	.	+	+	−
X	1	+	+	+	+	+	−	−	−	+	Pale yellow	7	Saccate	+	+	−
XI	1	+	+	+	+	+	−	−	−	+	White	7	Saccate	+	−	−
XII	4	+	+	+	+	−	−	−	−	−	Fawn to orange	8	Saccate to infundibuliform	+	+	−
XIII	1	+	+	+	+	+	−	+	−	+	Orange	.	.	+	−	−
XIV	1	+	+	+	+	−	−	−	−	+	Orange	7	Saccate	+	−	−
XV	1	+	+	−	−	+	−	+	−	−	Chalk white	7	Stratiform	−	+	+

Table VI. *Classification of the streptococci isolated from rennet extract*

Group	No. of cultures	Carbohydrate fermentation								Nitrate reduction	Pigment	Gelatin liquefaction		Litmus milk		
		Dext.	Sucr.	Lact.	Malt.	Mann.	Inul.	Salic.	Raff.			Time days	Form	Acid.	Coag.	Pept.
XVI	2	+	−	+	+	−	−	+	−	−	White	.	.	+	−	−
XVII	2	+	+	+	+	+	−	+	−	−	White	.	.	+	+	−
XVIII	1	+	+	+	+	+	−	+	+	−	White	6	Saccate	+	+	+

in older samples. The flora of the 2 weeks old sample of rennet consisted mainly of acid-forming orange cocci, and it is reasonable to expect that these types were derived from the original "vell". From the three samples examined, cultures of staphylococci, *Micrococcus caseolyticus*, *M. freudenreichii* and *Streptococcus liquefaciens* were isolated among others, and it is interesting to note that similar types were found in the foremilk. Since the "vells" for rennet manufacture are obtained from milk-fed calves, it is possible that a number of udder organisms gain access to the stomach of the calf *via* the milk suckled from the dam. These udder types would in all probability be suited for existence in the calf's stomach, since such types would be resistant to the bactericidal action of gastric secretions.

Throughout the work of classifying the cocci isolated from rennet, it was noticed that many of the cultures showed considerable variation in their biochemical characters. For this reason it was difficult to assign names, and further to group similar strains together. In several cases it was found that one culture liquefied gelatin, a similar one did not or did so very slowly. Sugar broth fermentation was likewise variable in similar cultures. It is possible that this variation was due to the prolonged action of the salt affecting the metabolism of the bacterial cell.

It appears that the flora of rennet extract can exert its influence in the ripening of cheese in one of two ways. Firstly, bacterial types are present which if they persist during ripening would aid in the final protein degradation. These include acid-proteolytic cocci and proteolytic cocci. Secondly, the rennet also contains comparatively large numbers of contaminating types such as moulds, yeasts and actinomyces. These organisms are a potential danger to the cheese maker and may possibly result in a deleterious effect under suitable conditions.

SUMMARY

A survey has been made of the flora of foremilk and rennet extract with special reference to acid-proteolytic types. In both instances, acid-proteolytic cocci were isolated of the type *Staphylococcus* sp., *Micrococcus caseolyticus*, *M. freudenreichii*, *Streptococcus liquefaciens*. Older rennet samples were found to be more productive of proteolytic cocci than either the fresh rennet or the foremilk. Many of the isolated cultures from rennet and from the foremilk failed to show any obvious peptonisation in milk, but yet were capable of liquefying gelatin. These strains may aid in the final breakdown of split-protein products in a medium such as ripening cheese. The isolated acid-proteolytic types were capable of considerable casein degradation in milk. In conclusion, it appears that the foremilk and rennet extract contribute a number of acid-proteolytic organisms to cheese, and that these types may aid in the ripening process.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION C. DAIRY CHEMISTRY

THIS review records the progress of dairy chemistry for the period extending from the end of that dealt with in a previous review(1) to August 1935; the subject will be dealt with under the same broad subdivisions as in the previous review.

No new journals describing researches into dairy chemistry have appeared; a second edition (1935) of *Fundamentals of Dairy Science* by Associates of L. A. Rogers (Reinhold Publishing Corporation, New York) has been published.

In the progress of dairy chemistry during the last two years, the subjects which have aroused greatest interest are: the composition and autoxidation of butterfat; the composition of casein; the physical chemistry of milk and its products; and the technology of butter and cheese manufacture.

I. THE COMPOSITION OF MILK

(*a*) THE AVERAGE COMPOSITION OF MILK

In the following table are collected additional data on the average composition of milk:

Investigators	No. of samples	Fat %	Solids-not-fat %	Country
Golding *et al.*(2)*	106	3·82	8·86	S. England
Golding *et al.*(2)†	1498	3·82	8·81	,,
Cornalba(3)	—	3·75	8·80	N. Italy
Rogina(4)	379	3·86	—	Balkans

* Calculated from the results for morning samples taken on one day per week.

† Calculated from the results for morning and evening samples taken every day for roughly 2 years.

In addition to the average proximate composition of milk given above, Cornalba(3) found an average of 4·8 per cent. of lactose, 2·8 per cent. of casein, 0·46 per cent. of albumin and 0·8 per cent. of ash. Rogina(4) found the fat content of his 379 samples to vary from 2·71 to 4·81 per cent., the standard deviation from the mean being 0·95.

Cole and Johansson(5) found that the composition of the milk of Aberdeen-Angus cows compared favourably with that of the milk of the Channel Island breeds, *e.g.* 4·06 per cent. fat and 3·56 per cent. protein, both decreasing slightly with advancing age of the cow. There was also a correspondingly high lactose content.

The cases of adulteration of milk, reported by the Ministry of Health(6) annually, account for roughly 7·5 per cent. of the samples examined by public analysts. The figures are:

1932–33: 5307 out of 72,940 samples (7·3 per cent.);
1933–34: 5760 out of 74,545 samples (7·7 per cent.).

(*b*) COMPOSITION OF THE MILK OF OTHER SPECIES

The following compositions of milk of mammals other than the cow have been recorded:

Sow(7): fat, 6·77; total solids, 17·98; protein, 6·22; ash, 0·97 per cent. Ca and P of colostrum, 0·076 and 0·083 per cent. respectively.

Buffalo(8): fat, 7·19, solids-not-fat, 9·43 per cent.

Nottbohm and Philippi(9) have reported that the chloride content of goat's milk is twice that of cow's milk, *i.e.* from 0·130 to 0·190 per cent.

II. THE CHEMISTRY OF MILK CONSTITUENTS

(*a*) Milk-fat

(1) *Composition of cow's butterfat.* Considerable attention has been paid to the unsaturated acids of butterfat, particularly with respect to the types of acid present and the factors influencing their amount in butterfat. Bosworth and Brown(10) and Eckstein(11) have examined the unsaturated fatty acid components of butterfat. The former investigators subjected the methyl esters of the fatty acids of a typical sample of creamery butter to exhaustive fractionation (37 fractions) and each fraction was examined for unsaturated acids by derivation from the iodine value. They proved the presence of decenoic ($C_{10}H_{18}O_2$) and tetradecenoic ($C_{14}H_{26}O_2$) acids, but the evidence for the presence of hexadecenoic and eicosenoic acids was doubtful; they failed to verify the presence of linoleic acid but suspected the presence of either a C_{20}, C_{22}, or a C_{24} acid containing two double bonds. Highly unsaturated acids of the C_{22} series, probably of the arachidonic type, were found to occur in butterfat, while from the four highest fractions, a mixture of saturated acids of high molecular weight, principally lignoceric acid with small amounts of behenic and cerotic acids, was isolated.

Eckstein calculated the linoleic and linolenic acid contents of a representative sample of butterfat, produced in Michigan, from the weights of the crystalline bromide fraction. He found that the linoleic acid content varied from 0·17 to 0·25, and linolenic acid content from 0·07 to 0·17 per cent. of the butterfat; these values were also probably too low. The linolenic acid content of butterfat could be increased by feeding linseed meal. Bosworth and Sisson(12) could not substantiate these findings, but isolated arachidonic acid as its octabromide. By their method of exhaustive fractionation of the methyl esters, they were again unable to detect the presence of linoleic and linolenic acids.

Dean and Hilditch(13) have summarised the factors which influence the distribution of the fatty acids of butterfat. Two main characteristic changes have been evident: (*a*) The change due to *season* is reflected in an abrupt increase in the proportion of oleic and linoleic acids with a parallel diminution in butyric and stearic acids. The return of cows to pasture in spring causes an immediate increase of 4 per cent. in the molecular percentage of the unsaturated acids, the diminution in saturated acids being wholly shared by butyric and stearic acids. (*b*) The change due to the *age* of the cow is reflected in a gradual increase in the amount of unsaturated acids over a period of years and a decrease in the content of palmitic acid; the change runs parallel to that observed for beef tallows, whose contents of palmitic and C_{18} acids decrease with the advancing age of the animal providing the depot fat.

The seasonal variations in the composition of various butterfats of Continental origins have been studied by Huyge(14) for Belgian, and by Peter and Kion(15) for Hungarian butters. The former found the volatile fatty acid content to be highest in April and May, with another subsidiary peak in October and November. The latter workers found that the Reichert-Meissl values were lower in the summer months than in the spring and early autumn, and that plenty of green material in the food of the cow always caused the butterfat to show high R.-M. and Polenske values. The refractometric readings were lower (40·5–42·0) in winter than in summer (42·0–44·5). They found that the iodine value was connected with the refractometric

reading by the following expression: Iodine value = 4 × (refractometric reading (at 40° C.)–40·4) + 26. They encountered a variation in iodine value of from 26·6 to 46·0.

(2) *Composition of butterfat from other mammals.* Dhingra(16) has studied the composition of the butterfat of Indian goats and sheep. The fats of the milk of these two species are similar in composition but differ from that of the milk of the cow or buffalo by showing a higher content of the lower fatty acids. Their content of fully-saturated glycerides appears to be a function of the mean unsaturation. The general properties of the component glycerides are the same as for those of the fat of the cow or buffalo. Butters made from sheep and goat's milk show a low R.-M. value (23·4–23·8), but otherwise follow cow's butter closely in their range of constants(17).

Dhingra(18) has also found that the amount of volatile fatty acids in the milk-fat of the Indian camel is lower than in that of the cow, buffalo, goat and sheep.

Schmidt-Nielsen and Frog(19) have shown that the fat of whale milk has an iodine value of 139, due to 38 per cent. of the fat being made up of C_{20}–C_{22} acids containing from two to six double bonds. There are also present 32 per cent. of C_{18} acids and 22 per cent. of C_{16} acids.

(3) *Autoxidation of butterfat.* Considerable progress has to be reported in our knowledge of autoxidation of milk-fat, particularly with respect to off-flavours in liquid milk. Kende(20) has reiterated the well-known fact that oxidised flavours in milk are due to the catalytic action of traces of heavy metals on the oxidation of finely divided milk-fat. The tendency to give oxidised flavours is variable, owing to the food of the cow adding varying amounts of protective substances to the milk. Milk heated to 80–85° C. is protected owing to the formation of some reducing substances in the process. The effect of traces of copper varies with the amount, from 2 to 4 parts per million giving the maximum off-flavour. Kende also postulates the occurrence of an enzyme—*oleinase*—as a causative agent in off-flavour development. The mechanism of this reaction is obscure, since the enzyme is not destroyed by pasteurisation. Kende is of the opinion that traces of elaidin give the tallowy taste.

Thurston, Brown and Dustman(21) express the opinion that lecithin rather than butterfat is the constituent affected when an oxidised flavour develops.

The advanced autoxidation of traces of lecithin is usually accompanied by fishiness, a taint which has not been known to occur in *milk* solely as the result of fat autoxidation. The oxidised flavour of milk is not identical with tallowiness of fats, since the separated fat of oily milk does not give positive reactions in the delicate tests for incipient fat autoxidation. The cause of the oily flavour therefore may be traces of either fat peroxides, or possibly hydroxy acids formed by the saturation of the double bonds of the unsaturated acids.

Csiszar(22) postulates two causes of oxidised or emery flavour, namely, that (*a*) the autoxidation of butterfat will cause a flavour which is indistinguishable from (*b*) an oily rancidity due to fat hydrolysis (or possibly the secondary effects of hydrolysis). This explanation was offered in polemical controversy against Kende's oleinase-heavy metal conceptions(23).

The possibility of the development of an oxidised flavour in milk in the absence of catalysts has been advanced. Guthrie and Brueckner(24) have investigated this by taking from individual cows samples of milk not exposed to metallic contamination. During a 3-day storage period, they found that 21 per cent. of milk samples from 155 cows developed an oxidised flavour. There was no correlation between breed, stage of lactation or age of cow with the incidence of the taint. The taint appeared in the fore-, mid- and last-drawn milk; the taint was more intense in winter than in summer, but the connection with the food of the cow was obscure.

Pasteurisation at 71° C. and higher for 30 min. decreased or prevented the incidence of the taint.

Kende(20, 23, 25), however, maintains that the substances which protect milk-fat from becoming spontaneously autoxidised are transferred to the milk from green foods, especially meadow hay and grasses; concentrated foods are poor in these factors. The protective factors are similar to the vitamins in their quantity and specificity; thus, ascorbic acid, in amounts of from 0·5 to 1·0 per cent., is claimed to be protective.

The reducing substances formed by the bacteria of milk together with their oxygen demand are also protective factors. Kertesz(26) states that the protective substances present in a dry preparation of *Reductobacterium frigidum*, which can be added to milk or cream in the proportion of 1:25,000 to prevent the development of oxidised flavour, are solely products of bacterial metabolism. Ritter and Christen(27) found that the material did not reduce methylene blue but gave the Tillmann's reaction for ascorbic acid. They also found 5–7 per cent. of hydroquinone as an adulterant in the preparation.

Krenn(28) reports that 0·3 part per million of added copper will cause milk to develop an oxidised flavour. Strawberry ice cream is specially susceptible to tallowy flavours. Tracey *et al.*(29) state that the elimination of copper is the most necessary step in avoiding the taint. They, however, find that strawberries contain two opposing properties controlling the taint development, one, a catalyst contained in the juice (also present in citrus fruits and in pineapple) and the other, a retarding agent associated with the fibrous material. Ross(30) states that the factors influencing the development of talloviness are associated more with the milk product than with the fruit, and that careful selection of the condensed or dried milk is the best means of prevention; the fruit masks rather than intensifies the taint.

(*b*) Lactose

The physical chemistry of lactose has been extensively studied by Herrington(31). Aqueous solutions of lactose, saturated for the temperature range 46–96° C., either alone or in 0·7 per cent. agar, were allowed to crystallise at various degrees of supercooling without agitation. Less supercooling was found necessary for the more concentrated solutions, but there was no sharp line of demarcation between the metastable and labile states. As the temperature of crystallisation was lowered, the rates of crystal formation and of crystal growth passed through maxima (about 30° C.). Lactose, precipitated from solution by ethyl alcohol, was of varying composition.

The crystalline habit of α-lactose hydrate varied greatly under different conditions of crystallisation. The principal factor governing the crystalline habit was the rate of crystal growth, or "precipitation pressure", which could be measured by the ratio of the actual lactose concentration to the solubility. The influence of sucrose on the crystalline habit was due to its precipitating effect on lactose. Sufficiently rapid crystallisation of both α-hydrate and β-anhydride formed needles, those of the former being straight and those of the latter curved.

The rate of loss of water of crystallisation of the α-hydrate is slow at 80° C., but the rate is greater with small crystals than with large; crystal size, however, is of little importance when the β-anhydride takes up water.

The mutarotation of lactose, generally attributed to catalysis by $H^{\cdot}$ and OH', is accelerated by molecules and by ions other than $H^{\cdot}$ and OH'. The catalytic effect of the anions of weak acids is much greater than that of the cations of weak bases. Thus the catalytic effect of lactate ion is small below a concentration of 0·1 *N*, but increases rapidly at higher concentrations. The effect of the other salts in dairy products is very small.

In determining the effect of the solvent on rotation, it has been found that there is more of the high than the low rotatory form of lactose in glycerol solutions. Therefore the specific rotation of lactose is increased in glycerol solutions, while it has been found to be decreased in alcohol and acetone solutions. It may be assumed that water is not an important factor in determining the ratio of the sugars at equilibrium. In the presence of salts, the specific rotation of lactose is altered; changes in the concentration of lactose or of the salt cause a change in the equilibrium rotation, this pointing to molecular compounds being formed in salt solutions.

For the preparation of β-lactose, Supplee and Flanigan(32) suggest the rapid drying of a film of lactose solution on a hot surface at such temperatures above 100° C. and for such a time that about 2 per cent. of moisture only remains; the heat remaining in the film must be sufficient to dry the product and maintain the crystallisation of β-lactose and not the α-lactose anhydride, *i.e.* such as not to allow the temperature to be lower than 93° C. during crystallisation.

Webb(33) has studied the factors associated with the browning of lactose in heat-treated products. The phosphate radical has a specific effect in causing the browning. The brown colour is more marked with increase in concentration of hydroxyl ions, amino acids, ammonium salts and oxygen. Traces of heavy metal salts retard, while sodium bisulphite prevents, colour formation; traces of formaldehyde cause a deeper brown to develop, while large amounts prevent the development of any colour. At the *p*H of milk, both reactions which are considered necessary for browning to develop, namely, humin formation, from the action of amino acids on sugar, and the formation of lactocaramel, can occur. Webb states that no effective means can be found to prevent the slight browning of evaporated milk, but that the shortening of the storage period and the lowering of the storage temperature will tend to make the fault less evident.

(*c*) Proteins

(1) *The composition and properties of casein.* The non-homogeneity of casein has been further studied by Lindestrøm-Lang(34) and Cherbuliez and Meyer(35). Casein is a mixture of two or more fractions which can be separated by treatment with acid-alcohol; the fractions differ in their chemical composition, especially in their phosphorus content. The former worker, investigating the solubility of casein in acids and bases, has found that the solubility is a function of the amount of casein present as precipitate and that the fractions in solution differ in chemical composition from the undissolved part of the precipitate.

Cherbuliez and Meyer separated casein into four components, α_I-, α_{II}-, β- and γ-casein, by precipitation from solution in alkaline ammonium chloride by the alternate addition of hydrochloric acid and acetone. Casein from six different sources and a freshly-prepared sample gave widely different amounts of these fractions under identical methods of separation; this was taken as evidence that the fractions were original components of the casein and not degradation products. The α_{II} fraction is responsible for the coagulation of the casein complex with rennin.

The mode of combination of the relatively high phosphorus content of casein has been further investigated. The phosphorus-rich peptone, glutamyl serine phosphate, has been isolated from tryptic digests of casein by Grabar(36) and by Levene and Hill(37). The latter workers precipitated the phosphopeptide from the digest with neutral lead acetate and isolated it as the brucine salt. The N/P ratio in the peptide was 4. Lipmann(38) has confirmed the linkage of the phosphoric acid to serine in the dipeptide.

Jones and Gersdorff(39) have drawn attention to the destruction of the cystine of casein by the action of strong alkali, especially in casein purification processes.

The cystine content of casein precipitated five times from sodium hydroxide solution is only one-tenth of that of the original casein. About 20 per cent. of the cystine of casein is liberated by 20 per cent. hydrochloric acid in 30 min., and the whole in 6 hours. Pepsin does not liberate cystine from casein. The lysine content of casein is not affected by either dry heat or alkali, but heat and racemisation with alkali may bring about a molecular rearrangement which renders part of the lysine precursors resistant to enzyme action(40). The cystine content of deaminised casein (0·27 per cent.) has been found to be identical with that of the original casein(41). Vickery and White(42), using improved methods, have redetermined the distribution of the basic amino acids of casein. Their figures for Hammarsten casein are: histidine, 1·83; arginine, 3·85; lysine, 6·25 per cent. Tomiyama *et al.*(43) have found 0·41 per cent. of methionine in dry ash-free casein. Casein forms salts with strictly equivalent amounts of organic bases(44).

(2) *Other milk proteins.* A. H. Palmer(45) has concentrated casein-free whey by freezing, from which he separated lactoglobulin by precipitation with sodium sulphate. On dialysis, he was able to isolate the crystalline globulin which was insoluble in water within the *p*H range 4·5–5·5.

The adsorbed protein on the fat globules of cream has been found by Wiese and L. S. Palmer(46) to have a high arginine, and low histidine and cystine contents. The protein is not identical with casein, albumin or globulin but resembles haptein.

Both Davies(47) and Jones and Little(48) have observed the different albumin and globulin content and distribution in abnormal milk when compared with normal milk. There is more albumin and globulin and a different albumin/globulin ratio in abnormal milk.

(*d*) Ash

The seasonal variation of the ash constituents of milk, as investigated by Elser(49), consists of a greater content of inorganic salts in winter than in summer milk since phosphoric acid and sodium salts are at their maximum percentages in winter. The calcium is also at its highest in winter and decreases gradually till the summer, but the Ca/P ratio is higher in summer than in winter. The Na/Cl relationship in milk is linear and can be expressed by the equation:

$$\text{Cl (mg. per 100 ml.)} = \text{Na (mg. per 100 ml.)} \times 1{\cdot}24 + 18{\cdot}1 \text{ (50)}.$$

The readjustment or removal of calcium and phosphorus from milk by base exchange has been investigated by Lyman, Browne and Otting(51). The idea behind the study was to modify the curd tension of milk by decreasing the concentration of ionic calcium. The replacement of calcium by sodium ions from a sodium-zeolite decreased the titratable acidity, but this could be overcome by artificially increasing the acidity of the milk before treatment to 0·3 per cent. When 22 per cent. of Ca and P were removed from the milk, the ability to clot with rennin was lost, unless the milk was boiled, in which case better coagulation was obtained if mineral acids instead of weak organic acids, had been used initially to increase the titratable acidity. The Na/K ratio of the milk was not disturbed if the zeolite was revived with a solution of chlorides of Na and K in the proper ratio; reviving the zeolite with sodium hydroxide was necessary for the removal of phosphate. The ash of the treated milk was lowered, even if mineral acid was used for acidifying, but was increased when lactic or citric acid was used.

The quantitative aspects of the lowering of curd tension, the preservation of the phosphate content and the application of this method as a quick way of determining ionisable calcium in milk need further investigation, using zeolites as laboratory reagents.

Nottbohm and Philippi(52) have studied the biochemical relationship of the ash

components of the milk and blood for both the cow and the sheep. A critical chloride content of 0·2 per cent. in cow's milk marks a breakdown in the selective secretory activity of the alveolar cells with respect to the blood constituents. With chloride contents lower than the critical value, the Cl/Na ratio increases and the K/Na ratio diminishes while the K content is constant. Above a chloride content of 0·2 per cent., these ratios are irregular. The higher chloride content of normal sheep's milk causes the approximation of composition of the ash constituents to those of blood to be more marked.

(*e*) Enzymes

Toyama(53) has evolved a quick method of concentrating the *xanthine oxidase* of milk by taking advantage of the adsorption of the enzyme on the surface of the fat globules; the fat of a high-fat cream is dissolved out with ether and the residue, which is dried *in vacuo*, contains the oxidase in a concentrated form. The preparation has a low protein content (5·1 per cent. of nitrogen) and a high iron content (3·35 mg. per cent.). An albumin-like fraction of globulin is associated with the oxidase. The optimum *p*H of the enzyme is 8·0, and the optimum temperature 40° C. The enzyme is inactivated by alcohol, acetone, salts of Ag, Cu, and Hg, sunlight and ultra-violet light; ether, chloroform, toluene and glycerol do not inhibit the activity of the enzyme.

The native *lipase* of milk has been investigated by Csiszar(54), Ramsey and Tracey(55) and Hileman and Courtney(56). Its activity in frozen milk and its general fat-splitting properties, together with the fact that the rancid milk inhibits bacterial growth, have been offered as evidence that the enzyme is not of bacterial origin. Heating for 20 min. at 63° C. kills the enzyme, while concentrations of 0·2 per cent. fluoride and 0·1 per cent. hydrogen peroxide are toxic. Homogenisation of milk activates the enzyme. There is an antagonism between the enzyme and the factors which cause fat autoxidation in milk. The activity of the enzyme is seasonal, being most active in mid-winter, and least in June–July. The factors governing lipase secretion are connected with the lactation cycle, the amount generally increasing with advanced lactation and with udder-abnormality.

The *amylase* of milk has been studied only in the direction of using the activity of the enzyme to detect raw or improperly pasteurised milk. Kluge(57), working on the lead serum of milk, has found that raw milk has a mean diastatic value of 1·312 (g. of starch hydrolysed by 100 ml. of milk in 3 hours at 39° C. at *p*H 6·9). The activity is reduced to 0·15 by momentarily heating the milk to 75° C., and completely destroyed by boiling. Weinstein(58) finds that the enzyme system is bound to albumin or globulin.

The *phosphatase* of milk and milk products has been studied by Kay and Graham(59). It is completely destroyed in the holder-pasteurisation process (see Section V, below).

(*f*) Minor constituents of milk

(1) *Phosphorus compounds.* The distribution of the phospholipids of milk has been studied by Perlman(60) who found that by removing cream of 15–20 per cent. butterfat content from milk, about 40 per cent. of the phospholipids remained in the skim milk, but that with increasing fat content (up to 58 per cent.) of the cream, the phospholipid content of the cream increased uniformly, after which it diminished. There is no evidence that heat alone destroys the phospholipids of milk, as has often been reported, but some milk bacteria secrete an enzyme which can hydrolyse these compounds. Mohr *et al.*(61) confirm the stability of milk lecithins towards heat. They note the lack of concordance of the values given in the literature for the phospholipid contents of various milk fractions, and, by improved methods of isolation (extraction with absolute alcohol and chloroform), find the following contents:

skim milk, 0·016; whole milk, 0·037; cream, 0·169; butter, 0·206; whey (mostly in the coagulated casein), 0·007 per cent. Bird *et al.*(62) find that 25 per cent. of the fat of buttermilk is phospholipid, corresponding to 0·152 per cent. in the buttermilk. Holm *et al.*(63) describe a nephelometric method of determining phospholipids. In the Babcock test for fat in buttermilk, only traces of the lipids appear in the fat layer, but in the Roese-Gottlieb method from 6 to 17 per cent. is extracted. They find the phospholipid content of skim milk to be 0·13 and, of buttermilk, 0·27 per cent.

Demair and Bleyer(64), who extracted the phospholipids from dried milk with methyl alcohol, found a monophosphatide containing palmitic, stearic and oleic acids together with choline and colamine. Kurtz *et al.*(65) found that the lecithin and kephalin fractions of milk phosphatides contained none of the lower acids and no palmitic acid. The amounts of fatty acids in these fractions were found to be: myristic, 5·2; stearic, 16·1; oleic, 70·6; arachidic, 1·8; C_{24} acid, 6·3 per cent.

The phosphorus partition in milk has been studied by Kay and Graham(59), who have evolved a simple method of separation. They have concentrated on the fate of the acid-soluble ester-P by the action of phosphatase. The amount of ester-P is from 0·15 to 0·25 of the total inorganic P, *i.e.* from 7 to 21 mg. P/100 ml. Hochheimer(66) has tabulated the phosphorus distribution of goat's milk for one lactation. The acid-soluble ester-P averages 8 mg./100 ml. of which 3·3 mg. is hexosephosphate P; hexosepyrophosphate, adenosine and creatine phosphoric acid account for the remainder.

Lampitt and Bushill(67) have found that heating or acid dissociation causes a slight alteration of the amount of dialysable phosphorus. About 40 per cent. of the total P, 50 per cent. of the inorganic P, and over 20 per cent. of the organic P are dialysable.

(2) *Heavy metals.* Blumberg and Rask(68) and Drea(69) give a list of the elements which they have detected spectrographically in milk. Both failed to detect Mn, Al and F in milk, although these elements, in Drea's case, were present in the food and water. Büttner and Miermeister(70), however, find a range of 0·06–0·17 p.p.m. of manganese in milk. The zinc content of colostrum has been found to be three times that of milk (3·4–3·6 p.p.m.).

The latest work shows that the values of the copper and iron contents of milk are lower than those found by earlier workers. Krauss and Washburn(71) report a copper content of 0·14–0·17 p.p.m., and an iron content of 0·40–0·53 p.p.m. and Remy(72) (for German milk), 0·13 p.p.m. of copper and 0·50 p.p.m. of iron.

Davies(73) has investigated the mode of combination of traces of heavy metals in dairy products; he finds that practically all the metal is in protein combination with about 1×10^{-6} in the ionic state. Increased acidity of sera causes slightly more heavy metal to enter into the ionic state. There is a tendency for heavy metal to concentrate on the surface of fat globules.

(3) *Whey pigments.* Kuhn and his associates(74) have isolated *lactoflavin*, $C_{17}H_{20}N_4O_6$, M.P. 276° C. (yield: 20 mg./litre) from whey. The pigment is identical with hepatoflavin and similar to ovoflavin. This flavin is either very concentrated vitamin B_2 or even the vitamin itself.

III. THE PHYSICAL CHEMISTRY OF MILK

(*a*) Physical properties

Lyons and O'Shea(75) have observed that the number of fat globules below 2μ in diameter increases and that of globules above 4μ decreases as the stage of lactation advances. An underestimation of the fat in the Gerber method by 0·1 per cent. can occur with milk in advanced lactation, and spinning three times is advocated.

No change in the viscosity of milk on adding lactic acid or by natural souring occurs until the titratable acidity reaches 0·45 per cent. lactic acid, after which there is a marked increase. The addition of rennet to milk first causes the viscosity to decrease and then to increase rapidly with curdling. Skim milk has a lower viscosity than whole milk and the viscosity of raw milk increases more during cooling than is the case with skim milk(76).

Trout(77) has studied the effect of homogenisation on the properties of milk. Homogenisation pressures of more than 1500 lb./in.^{2} are unnecessary to prevent a cream layer forming. Homogenisation has no effect on specific gravity but increases the foaming tendency of raw milk and decreases that of pasteurised milk. There is a decrease in viscosity observed after homogenising pasteurised whole milk. If the cream of milk, homogenised at pressures below 1500 lb./in.^{2}, is separated centrifugally, the fat loss in the skim milk does not exceed 30 per cent. Butter from homogenised cream shows no defects in working or in its texture. The homogenised cream, however, churns more completely when ripened than when not. On freezing homogenised milk there is very little separation ("oiling off") of fat.

The physical effects of freezing milk and cream have been examined by Webb and Hall(78). Slow freezing causes a gradual precipitation of the caseinate system and an immediate destruction of the fat emulsion. The heat stability is not affected unless the milk is held at $-18°$ C. for several months. Concentration, or increase in solids-not-fat content, and dissolved sugar lessen the destruction of the fat emulsion, and homogenisation retards slightly the separation of fat from low-fat creams. Freezing destroys the fat clumps formed in cream by homogenisation, and restores the heat stability. Condensed pasteurised whole milk can be frozen without loss of body or flavour and can be satisfactorily reconstituted after 4 weeks in the frozen state. Pure casein dispersions and milk serum can be prepared by freezing homogenised cream of 25 per cent. fat content; on slow thawing at temperatures below the melting point of the fat, a clear serum separates; the residue is washed with ice-water to remove the serum. On warming the mixture in water, the fat "oils off" leaving a casein dispersion which possesses all its original characteristics.

A method of increasing the viscosity of cream, advocated by Dahlberg and Hening(79), consists of warming the cold cream to 80–85° F. and cooling slowly to 40° F., the cooling for the last 30° taking about 2 hours; this viscosity is temporarily lost on heating to 60° F. but gradually returns. Excessive fat clumping does not appear to occur, but it is suggested that a higher hydration of the proteins is effected. The creaming properties of milk heated very rapidly to pasteurisation temperature and then cooled very rapidly are not changed(80).

Reid and Eccles(81) have observed the impairment of the whipping properties of cream by the addition of dry skim milk powder, since the titratable acidity and the viscosity are increased; there is no effect on the surface tension. Dry skim milk also increases the foaming properties and viscosity of liquid milk. According to Supplee *et al.*(82), the substances contributing to the foaming tendency of milk are contained in the group of "nitrogen extractives" which are associated with milk protein.

The failure of ice-cream mixes, made from butter and dried skim milk, to whip properly has been explained by Watts and Dahle(83) as due to the absence from the mix of the lecithin-protein complex which is present in cream. The addition of 0·5 per cent. of dried egg-yolk or sweet buttermilk is beneficial in restoring whipping-properties.

In the Schern-Gorli test, the rise of the carbon (or other finely divided material) varies directly with the rate of rise of the fat globules and indirectly with the adsorptive capacity of the globulin on the surface of the fat globules. Factors which

influence the dispersion of the globulin, *e.g.* shaking, change of *p*H or increase in electrolyte content, cause the adsorptive action either to decrease or disappear. The hydration of the globulin in pasteurised milk causes no positive test to be given(84).

The following table summarises the information on the average freezing point of milk:

Investigator	No. of samples	Average Δ	Range of Δ
Stubbs(85)	1000	0·544	0·529–0·563
Denis-Nathan(86)	Large number	0·541	0·528–0·561
Krenn(87)	712	0·545	0·513–0·582
Golding(88)	91	0·552	0·536–0·564

Krenn(87) has found that the electrical conductivity of milk varies from 38·0–68·4 $\times 10^{-4}$ mhos., 91 per cent. of samples falling between 41 and 50 $\times 10^{-4}$ mhos. The conductivity of milk from various species, according to Montefredine(89), is lowest for human and equine milks (16–17 $\times 10^{-4}$ mhos) and highest for sheep and goat's milk (56–60 $\times 10^{-4}$ mhos). High lactose milks (or low chloride) have low conductivity and *vice versa*.

(*b*) Heat coagulation

Rowland(90) has studied the effect of temperature and time of heating on the denaturation of the albumin fraction of milk. Heating milk for 10 min. at 63° C. causes 10·4 per cent. of the albumin fraction to be coagulated and, for a range of temperatures up to 75° C., there is a regular increase in the amount of denaturation. The velocity of denaturation over the range 63–75° C. for every 1° rise is constant; the temperature coefficient of the denaturing reaction is 1·5 per 1° C.

Howat and Wright(91) have studied the role of cleavage of the phosphorus during the heat coagulation of casein. They have found that when a neutral solution of calcium caseinate is heated to 120° C., the casein rapidly loses phosphorus and is partly degraded, and then the partly-dephosphorised product is coagulated; the base-binding power of the product is less than that of the original casein and calcium is also liberated in the heating process.

(*c*) Rennet coagulation and curd tension

Tauber and Kleiner(92) have proved that rennin is a true protein, and not an enzyme adsorbed on a protein carrier; rennin is inactivated by pepsin and trypsin but not by erepsin. Milk is not clotted by concentrated trypsin solutions but only by concentrations of 0·1 per cent. or less; after exposure of milk for 10 min. to solutions too concentrated to clot, the milk cannot be coagulated by active rennin preparations. This, however, can be avoided by bringing the *p*H of the milk to the acid side so as to avoid hydrolysis. With pepsin, trypsin and rennin, the velocity of milk coagulation increases with the $H^{\cdot}$ concentration. The addition of crystalline urease to milk inhibits the clotting action of rennin and pepsin; this is a property of the enzyme added, since boiling the urease solution destroys its effect. Blood serum and the mucosa of the pig's stomach and of the fourth stomach of the calf contain inhibitors which act differently on rennin and pepsin. Tauber(93) suggests this as additional evidence that the two milk-clotting enzymes, rennin and pepsin, are distinct enzymes.

Wuster(94) has examined the conditions governing the separation and drainage of whey from freshly coagulated rennet-curd. The character of the surface of the vessel is of importance since, if the curd clings to the surface, the rate of elimination of whey is reduced. Syneresis is increased at the milk/air and the milk/milk interfaces and by an increase of acidity. The acceleration of drainage occurs until a curd-calcium concentration of 0·6 per cent. is reached, above which the rate decreases. Increasing the temperature of the system increases the rate of drainage; heating of the milk

to 63–65° C. for 30 min., or to 70° C. momentarily, before cooling and renneting, diminishes slightly the rate of drainage. Conversely, the swelling of rennet curd is diminished by increased acidity, by a concentration of curd-calcium below 0·6 per cent. and by an increase of temperature during and after coagulation.

Doan and Welsh(95) state that soft-curd milk is characterised by low casein, low total protein, low plasma solids and low ash content. The curd tension of milk is a linear function of the casein content and can be calculated from the casein nitrogen content. The other constituents of milk have little effect. Curd tension is lowest in early summer and highest in early winter; it is highest in the first 4–6 weeks of lactation; the dilution of milk with water reduces the curd tension in proportion to the casein content. Owing to the dispersal of fat clusters by heating fat-rich milk, the curd tension is increased. Homogenising reduces curd tension, the optimal effect being obtained by heating to 180° F., cooling to 100° F. and homogenising at pressures not less than 2000 lb./in.^{2} The addition of alkali to milk reduces curd tension in proportion to the increase in *p*H. The Hill test does not give the maximum curd tension, but the best results are obtained by a pepsin-HCl mixture which gives a serum *p*H of 6·0–6·1. Udder infections decrease curd tension, but a low curd tension is no proof of udder infection.

The effect of homogenisation on curd tension has been investigated by Caulfield and Martin(96) who have found that the amount of decrease due to homogenising depends on the original curd tension of the milk. Homogenising at 165° F. gives a greater lowering than at 120° or 145° F. The change in curd tension is permanent and no lowering is effected by re-homogenisation. Berry(97) finds that holder-pasteurisation does not lower curd tension, but heating to 180° F. has a marked lowering effect as also has heating to boiling point. Freezing increases curd hardness but viscolising at 3000–5000 lb./in.^{2} lowers it greatly.

The excess of calcium chloride in the coagulating mixture advocated by Hill retards coagulation and gives subnormal values for the curd tension. Miller(98), therefore, suggests that a coagulant made up by dissolving 0·45 g. of pepsin in 100 ml. of 0·4 per cent. hydrochloric acid gives a truer picture; the calcium chloride precipitant shows a greater divergence in curd tension than the acid-pepsin coagulant.

Lundstedt(99) claims that, if the lecithin is removed from the surface of the fat globules by churning so that it is absorbed on the casein network, its property as a protective colloid will convert whole milk of high, to one of low, curd tension.

(*d*) Acid-base equilibria

The use of lactic acid instead of mineral acid in the determination of the buffer value of milk has been suggested by Okulitch and Golding(100), since a truer picture of the changes in milk is given by the weaker organic acid. There is, however, no difference in the values obtained by either acid. The variation in the *p*H of monthly samples of milk is considerable, Ayrshire milk varying less than Jersey. The buffer values vary in a similar manner. A change from indoor to outdoor conditions shows a definite lowering of the *p*H of the milk produced, and an increase in the buffer value of Jersey milk.

Small differences in the *p*H and titratable acidity of milk have been observed by Caserio(101) according to the metal of the vessel in which the milk is kept. The changes in *p*H on storage are greater when the milk is stored in impure aluminium or tinned iron. The purity of aluminium must not be below 95 per cent. for milk work.

The electrolytic neutralisation of milk has been examined by Gratz(102) and Winkler(103). The milk is made to flow between several pairs of charged electrodes and in this process the acidity is reduced. Gratz states that the lactic acid is reduced

to acetaldehyde and later changed to carbon dioxide and water. It is more likely to be neutralised by alkali hydroxides from the electrolysis of alkali chlorides, the chlorine entering into organic combination in the protein. The milk so treated is claimed to possess good keeping quality with no change in taste or smell.

(*e*) The casein complex

Brigando(104), who surveys our knowledge of the state of casein in milk, states that casein exists in milk as a colloidal complex with $CaHPO_4$; acid and rennet casein are obtained by action on this complex; acid casein is obtained by the change of $CaHPO_4$ to $Ca(H_2PO_4)_2$ and a progressive decalcification of calcium caseinate by added acids or by souring. Pure casein should be salt-free but this is never the case. If acid casein is prepared by precipitation at low temperature and is well-washed, first with water and then with acid water of *p*H 4·7, a low-ash product is obtained, but excess acid in the precipitation and vigorous stirring do not lower the ash content. Theoretically, rennet casein is an association of two salts—calcium para-caseinate containing entrained $CaHPO_4$. Renneting high-acid milk lowers the ash content of rennet casein. The neutralisation of acid milks before renneting gives rennet casein of normal ash content (about 7·5 per cent.). Rennet casein has a titratable acidity corresponding to 2·1 g. of lactic acid/100 g. of dry matter; the corresponding value for acid casein is 8·1 g./100 g. dry matter.

Acid, soft-curd, and heated milks differ in their proportions of the soluble and insoluble calcium phosphate which they contain; these differences can be overcome by the adjustment of the calcium and phosphate to the proper ratio. Acid milks, *i.e.* milks of high titratable acidity, are rich in calcium but low in phosphorus, while soft-curd milks are high in phosphorus but low in calcium(105).

Pyne(106) produces further evidence that the phosphate of milk is mainly tertiary and is in intimate union with calcium caseinate.

There is a measurable combination of calcium with casein when milk is boiled or when casein is boiled in a solution of calcium chloride (Ballowitz(107)); this is accompanied by partial precipitation. This heat precipitation is explained by the fact that casein is partly hydrolysed and that the amount of precipitation depends on the concentration of calcium (cf. Howat and Wright(91)). Casein boiled in acetate or phosphate buffers does not undergo partial heat precipitation.

IV. MILK PRODUCTS

(*a*) Butter

(1) *Technology of manufacture.* The reactions occurring during the neutralisation of sour cream with lime and sodium bicarbonate have been discussed by Wiley(108) from the point of view of acid-base equilibria. When lime is used as a neutraliser, pasteurisation has little effect on the *p*H and titratable acidity of the neutralised cream, but when sodium bicarbonate is used, the loss of carbon dioxide, the extent of which depends on the method of pasteurisation adopted, has most influence on the degree of neutralisation obtained; this influence varies with the original acidity of the cream. The reduction in acidity when lime is used deviates from that calculated by titration with caustic alkali to an extent depending on the *p*H of the neutralised cream. Thus when cream is neutralised to a *p*H of 5·8, only 75 per cent. of the theoretical reduction in titratable acidity is achieved; a neutralisation of the acidity to any *p*H above or below this value, however, gives a greater percentage of the theoretical reduction of acidity.

The *p*H of the serum of the best Australian butters has been found by Wiley(109) to vary from 5·2 to 7·8, most samples grouping within the range 6·3–7·1. The grading

of fresh butter could not be correlated with serum acidity. Arup and Gilmour(110) have found that butter from unripened cream (serum *p*H 6·75–7·55) stores better than butter from ripened cream (serum *p*H 5·60–6·75). Less curd also is found in the former group of butters. The *p*H of serum is regarded as a characteristic of more value in forecasting the keeping quality of butter than is the titratable acidity; butters for storage should have a serum *p*H of not less than 6·70. The curd content also should not exceed 0·3 per cent. and the air content, 3 ml./100 g.

The different factors influencing the hardness of butter have been further investigated. Wode(111) prepared butter under standard conditions, added 1 per cent. of salt and stored at 2° C. for varying lengths of time and measured the hardness at 15° C. The factors which tend to give hard butter are: chilling the cream before or after ripening, holding the cream at low temperatures, the rapidity of warming chilled cream before churning, low ripening temperatures, length of storage time, and cooling the butter before working. Greasiness and crumbliness depend on the amount of free fat and the degree of its crystallisation. Hardness is probably governed by the distribution of the free fat between the fat globules and the free fat, by the lubricity of the free fat and the firmness of the fat globules.

Under standard conditions of churning and working of butter, Coulter and Hill(112) have found that the hardness of butter bears an inverse relationship to the iodine value of the butterfat. The hardness is also influenced by the water content; the water content also has been found to increase with the iodine value. The churning time of cream is prolonged with increased hardness of butterfat; the fat losses in the buttermilk decrease with increase in the time of churning. A seasonal variation in the fat losses in buttermilk can be correlated with fluctuations in the iodine value and hardness of the butterfat(113). Udy(114) is of the opinion that the agitation of hot cream is the most significant factor causing fat losses in buttermilk under manufacturing conditions.

The distribution of the aroma-producing factor and its precursors in cream and butter has been investigated by Michaelian and Hammer(115). Fresh buttermilk regularly contained larger amounts of acetoin and diacetyl than the original cream. The ratios of acetoin *plus* diacetyl in buttermilk to that in cream ranged from 2 to 3·1 : 1. Butter contained small amounts only. Thus, in 52 churnings, the ratios of acetoin *plus* diacetyl in butter to that in cream was 0·032–0·218 : 1. The amount in the butter-serum was greater than that in the fat. Mohler and Herzfeld(116) state that butter of normal aroma contains from 2 to 4 parts per million of diacetyl. Cream contains three times as much and buttermilk contains appreciable quantities. Excess of citric acid in cream may produce butter of excessive aroma and flavour(117).

(2) *Keeping quality and faults.* The relation between the catalase content of butter and its keeping quality has been studied. Knudsen and Jensen(118) claim that the catalase activity is good evidence of contamination with foreign micro-organisms. They have observed that all butter of good keeping quality has a low catalase content, but that low catalase content does not necessarily mean good keeping quality. Butter with small water droplets stores better than that with large droplets, and conditions likely to give large droplets, such as the addition of salt and churning from acid cream, give butter of low keeping quality. Schmidt(119) has suggested a catalase test for butter. Butter from pasteurised cream contains no catalase except that from bacterial contamination; a low-grade butter containing no catalase is usually tallowy.

Loftus-Hills *et al.*(120) state that the catalase content (or enzyme content in general) is not of great importance in determining the keeping quality of butter. Variations in curd and salt content, amount of ether-soluble acids, and peroxide content are also considered of minor importance. Serum acidity (*p*H) and copper

content are the most important factors. Bouska(121) has observed that the average titratable acidity of good butter ranges from 0·02 to 0·05 per cent. lactic acid. With acidities above 0·1 per cent. the fat is unstable, and below 0·02 per cent. the protein is broken down.

Ritter and Christen(122) have investigated the development of fishiness in butter. Factors such as high acidity, high salt content, overworking, and traces of copper and iron, which are conducive to autoxidation of lecithin, are mainly responsible. They have, however, not explained the true mechanism of the development of the taint, since, although free trimethylamine can be isolated from the tainted butter, the base itself is not the sole cause of the taint. Davies(123), describing the mechanism of the development of a fishy taint in milk through the feeding of beet by-products, states that the taint is due to the coupling of the active oxygen of trimethylamine oxide to the double bond of the oleic acid of the fat, and that fishiness generally is a form of incipient fat deterioration brought about in this manner. Ritter has also confirmed previous knowledge on autoxidative changes in butter, namely, the progressive effect of increasing amounts of copper and iron on the development of tallowiness and the inhibition of autoxidation by metol, hydroquinone, and *Reductobacterium frigidum*; ascorbic and maleic acids, aldehydes and exclusion of oxygen do not inhibit autoxidation.

Schlemmer(124) and Maier(125) have observed that vegetable parchment excludes the wave-lengths of light which act on butter to give autoxidative taints. A proprietary parchment treated with a solution of a yellow colouring matter of vegetable origin was found to exclude light of all wave-lengths between λ2000–3540. Davies(126), investigating the effect of the relatively high contents of copper and iron (17 and 45 p.p.m. respectively) of vegetable parchment, has found that fatty foods in contact with such wrapping material do not develop autoxidative taints at a more rapid rate than when stored in glass and not exposed to light. It was observed that, under commercial conditions of packing fatty foods in transparent Cellophane, it was the depth of colour and not the actual colour of the wrapper which was, on the whole, of importance in preserving the fat from the destructive effect of light rays.

Barnicoat(127) describes two surface defects of boxed butter wrapped in vegetable parchment. Surface drying of the butter, due to the porous nature of the wrapper, causes the surface to assume a deeper yellow tint (primrose colour). An off-flavour of the surface layer (toppiness) is due to autoxidation of the fat, possibly through the increase of acidity in that layer from loss of moisture. Riddet *et al.*(128) have found that all types of vegetable parchment give primrose tint and toppiness; wax paper is also unsatisfactory, but good results are obtained by using parchment layers interleaved with metal foil as a wrapper. Wood taint can be prevented by spraying the inside of the boxes with casein and hardening with formaldehyde.

(*b*) Cheese

(1) *Chemistry of manufacture.* The composition of the whey draining from the curd during Cheddar cheese manufacture has been investigated by McDowall and Dolby(129). During cheese-making the concentration of lactate in the whey rises steadily while the titratable acidity remains constant. A sharp fall in the lactose content of the whey occurs when salt is added to the curd, at whatever stage the salt is added. This is due possibly to the liberation of bound water. After cutting the curd, lactose is more concentrated in the whey than in the original milk; apparently about 2 per cent. of the water of milk is bound, *i.e.* the amount of bound water in cheese curd is about four-fifths of its casein content. When 0·1 per cent. of sulphate is added to milk at the start, the addition of salt to the curd increases the sulphate concentration in the whey by 50 per cent.

There is a steady rise in the concentrations of Ca and P in the whey up to salting, after which there is a temporary fall, the rise and fall of Ca being more pronounced. The concentrations of Ca and P in the whey depend on the titratable acidity of the whey; in normal cheese, 60 per cent. of the Ca, and 57 per cent. of the P are retained in the cheese. An increase in the acidity of the milk before renneting increases the loss of minerals in the whey and lowers the Ca content of the cheese. The buffer values of whey are in accordance with the above analyses of the liquid; it is the lactate which buffers between *p*H 3 and 5, and the calcium phosphate at higher *p*H values.

During the souring of milk by starter cultures, lactic acid accounts quantitatively for the increase in acidity.

In the dry salting of Camembert cheese, Kieferle *et al.*(130) find that whey is expressed rapidly, 25 per cent. being removed in 15 min. and 75 per cent. in 45 min. The NaCl content of the whey decreases while the lactose, nitrogenous compounds, Ca and P in the whey increase progressively. Too early a salting of the cheese causes over-salting owing to excessive absorption. If salting is left too late, the dry surface absorbs too little salt and under-salting ensues. In the latter case, the whey is retained, causing a bitter, acid cheese. An increase in the temperature of the ripening room causes more whey to separate and too much drying of the cheese. The best temperature is 18° C. Salt powder of high density or fineness can cause oversalting. The lactose content of the cheese, which first depends on the whey content, later depends on the salt content.

McCammon *et al.*(131) state that hard cheese retains 80 per cent. of the calcium of the milk, and soft cheese, 20 per cent. The phosphorus varies with the protein content, the amount retained for each type of cheese being 38 and 37 per cent. respectively. Guittonneau and Chevalier(132) have found that cheese made from milk renneted at *p*H 6·4 retains 69 per cent. of the Ca and 57 per cent. of the P, while if coagulation occurs at *p*H 5·4 only 12 per cent. of the Ca and 37 per cent. of P are retained in the cheese.

The findings of McDowall and Dolby and of Kieferle point to the importance of studying the colloidal nature of cheese. In the coagulation of a hydrophilic gel, the structure of which is influenced by the degree of swelling and the hydration of the protein, water is the important dispersion medium, and the extent to which it is bound influences the properties of the gel and the progress of ripening.

(2) *Cheese-ripening.* This has already been dealt with fully in a previous review(133). Léoncini *et al.*(134) suggest the use of a cheese ripening coefficient, namely, the percentage of the total nitrogen which is water-soluble. This coefficient increases slightly with temperature at optimum humidity but decreases with low humidity; in an atmosphere of toluene or chloroform, the coefficient is lowered owing to the poisoning of bacteria and enzymes.

Koestler(135) finds that the tenacity of the curd of hard cheese increases during the first three months and then decreases. High acidity gives a hard cheese which later cracks. With Emmenthal cheese the percentage of lactose in the milk and in the fresh cheese influences the *p*H and the general course of ripening. Fine granules of curd retain more moisture in cheese than large granules.

(*c*) Concentrated and dried milk products

Howat and Wright(136) have studied the various factors which influence the solubility of milk powders. In their investigations on the influence of the temperature of reconstitution on protein solubility, they find that as the temperature is raised from 20–50° C., the solubility increases, and then shows a slight regular decrease from 50–100° C. The same behaviour is shown by whole or skim milk powder

except that the whole milk powder tends to show a lower solubility. Overheating milk protein in the dry state renders it temporarily insoluble but the solubility can be recovered by reconstitution at 50° C. The fraction of the protein which remains insoluble is that permanently denatured by moist heat. The decrease of solubility at temperatures of 50–100° C. is due to the heat treatment during reconstitution.

The degree of ionisation of electrolytes in concentrated milk products cannot be determined from conductivity data owing to viscosity effects on ionic mobility. For concentrates containing less than 34 parts of total solids per 100 of water there is a linear relationship between the freezing point and the content of total solids, but, above this concentration, lactose crystallises out and the freezing point depression increases at a lesser rate. No bound water is evident at a concentration of 65 parts of milk solids per 100 parts of water. There is a linear relationship between concentration of milk solids and hydrogen ion concentration.

V. THE CHEMISTRY OF MILK PROCESSING

(*a*) Metals and milk

The behaviour of copper and nickel towards milk under various conditions has been studied by Whitfield *et al.*(137). They have found (by losses in weight) that nickel corrodes more than copper at all temperatures, that the corrosion at 144° F. is greater for both metals than at 60° F., and that, at 144° F., the corrosion in milk saturated with oxygen is greater than in that saturated with air and less than in that saturated with carbon dioxide. The weight losses per unit time are greater for short, than for long, exposures. Copper gives an oily flavour, but nickel only occasionally. Davies(138) describes cases of metallic contamination of milk during processing. The copper content of milk may increase in certain cases to over 1·5 p.p.m. The lower limit of copper content necessary to yield an oxidised flavour in ordinary commercially-pasteurised milk, stored at 0–5° C. for 24 hours, is 1·3 p.p.m. Contamination with iron proceeds simultaneously with that of copper. The value of sampling milk at various points during the pasteurising process is stressed.

Mohr *et al.*(139) state that sodium silicate protects aluminium against the action of caustic soda but not of caustic potash. Alloys are attacked by alkaline phosphates only when there is a reactive metal, *e.g.* Zn, Sn, Al or galvanised iron, in the alloy, but when copper and nickel are present there is no corrosion. Chromates prevent the attack of calcium and magnesium brines on all metals except zinc; the brine encrusts on lead and iron. There is no action also on either solder or tinned surfaces(140).

Milk scale has been found by Schmidt(141) to contain fat, 6·25; lactose, 0·44; protein, 13; ash, 71·0; water, 1·0 per cent. The scale can be effectively removed by soaking with alkali at 70° C. for 5–10 min., and washing off with cold water.

(*b*) Detection of heat-treated milk

Bengen(142) suggests that as some albumin is coagulated at 65° C., the addition of solid ammonium sulphate gives a serum which remains clear at 70° C. and cloudy at 71° C. in the case of pasteurised milk, but which is cloudy at 65° C. with raw milk.

The Schern-Gorli test has been modified by Standfuss and Sörrensen(143) and by Pien and Baisse(144). The former dilute milk to three times its volume with normal saline and add laked sheep's blood corpuscles. A test is given which is positive for 10 per cent. of raw milk added to milk pasteurised at 63° C. for 30 min. The latter workers disperse 1 g. of indigo and 1 g. of casein in 100 ml. of water and add 2 drops to 5 ml. of milk. After standing for several hours, milk held below 65° C. for 30 min. gives a blue cream line, that held at 67° C. gives a white line, while that held at 66° C. gives a doubtful result.

The amylase test is unsatisfactory for determining the length of time during which milk has been heated. The absence of a violet colour indicates that the milk has been heated to 55° C. only. But Bengen and Bohn(145) and Leahy(146) state that the test is useful for detecting the addition of raw to pasteurised milk or for detecting insufficiently pasteurised milk.

Kay and Graham(147) have improved their original test(59) for the determination of phosphatase activity in milk by the colorimetric determination of the phenol liberated by the enzyme from disodium phenyl phosphate. They have found that the pasteurisation of mixed milk by holding at 145° F. for 30 min. has destroyed the phosphatase activity of the milk to such an extent that the depth of blue colour given by Folin's reagent for phenol under standardised conditions does not exceed 2·3 Lovibond blue units. They suggest two tests, one of which can be carried out in 30 min. and will detect any gross error of pasteurisation technique. A second and more searching test will reveal minor errors of processing, such as: heating for 20 instead of 30 min. at 145° F. and heating $1\frac{1}{2}$° F. below the minimum temperature of pasteurisation.

Eble and Pfeifer(148) have observed that diphenylthiocarbazone gives an intense colour only with milk heated to 85° C. The reagent is claimed to be able to detect 5 per cent. of milk heated to 85° C. in raw or holder-pasteurised milk.

(*c*) Other subjects

The separation of serum in bottled non-homogenised cream can be prevented by pasteurising the milk at 160° F. for 15 min., separating at 120° F. and standardising to the correct fat percentage with pasteurised whole milk(149).

Tufft(150) has revived the methods of concentrating milk by freezing. By partially freezing milk and centrifuging off the liquid portion, about 50 per cent. of the water containing only 0·6 per cent. of total solids can be removed.

Cream is more apt to develop a cooked flavour than milk during pasteurisation and it is therefore advisable to pasteurise cream at 62° C. The cooked flavour involves both the fat and the serum but is greater in the fat(151).

VI. THE ANALYSIS OF DAIRY PRODUCTS

(*a*) Determination of the freezing-point of milk

Monier-Williams(152) has devised a new apparatus for the determination of the freezing-point of milk. It embodies the following advantages: (*a*) heat exchange between the milk and the surrounding medium at the point of freezing is eliminated; (*b*) the correction for supercooling can be easily and accurately determined; (*c*) the final temperature is constant for several minutes; (*d*) air is drawn through the ether instead of being driven; (*e*) the only glass members of the apparatus are the thermometers and a two-way stop-cock.

Elsdon and Stubbs(153) report the results of 3000 determinations of the freezing-point of milk by the Hortvet and Monier-Williams methods. They suggest a modification of the Hortvet method embodying the Monier-Williams principle of withdrawing the layer of alcohol from between the milk tube and the cooling zone when the milk is at the point of freezing.

Stubbs(154) claims that there is no significant difference between the three methods of determining the freezing-point of milk (Hortvet, Raoult and Monier-Williams methods). If standard apparatus is used, no attempt should be made to correct for super-cooling and heat transference. The values will then be consistent and comparable. The apparatus should be standardised against a precision apparatus such as the Raoult.

For the preservation of samples intended for a freezing-point determination, Rüdiger(155) suggests the use of small amounts of formalin, mercuric chloride or mustard oil.

(*b*) Milk proteins

Buruiana(156) suggests a colorimetric method for evaluating milk proteins by digesting 1 ml. of milk with 6–7 ml. of concentrated nitric acid and, after neutralisation and making up to 50 ml., comparing against known casein or dichromate solutions. Offutt(157) removes the casein from milk by Waterman's method, neutralises the filtrate and precipitates the albumin by heating on a water-bath. The albumin content is calculated from the difference between the nitrogen contents of the filtrates before and after heating.

The factor for converting the formol titration value to milk protein has been found by Pyne(158) to increase progressively throughout the lactation period. There appears to be a marked increase in the whey protein factor due to variations in composition of the non-protein nitrogen fraction. The formol titration method can be used to determine casein by subtraction of the formol value of the whey from that of the whole milk.

Offutt(159) determines milk protein in milk chocolate by first extracting the fat, and dispersing the residue in 0·3 per cent. sodium oxalate and filtering. The casein is precipitated from the filtrate by acetic acid and the difference between the nitrogen of the casein-free and the original filtrates gives the casein-nitrogen; this value multiplied by 1·25 gives the total milk protein.

(*c*) Detection of thickening agents

Acid mercuric nitrate is not a satisfactory clearing reagent in the Stokes method for detecting gelatine in cultured buttermilk and in soft cheese. Certain degradation products of casein not precipitated by the mercury salt give copious precipitates with picric acid. If half the volume of the liquid of 20 per cent. trichloracetic acid is used, these products are removed and the picric acid test can then detect from 0·10–0·25 per cent. of gelatine(160). Braunsdorf(161) notes that yoghourt cream gives a positive result with the picric acid method in the absence of gelatine. If the serum of milk or other product is treated with cadmium chloride and hydrogen sulphide, the presence of larger quantities than 0·025 per cent. of gelatine will prevent the formation of a precipitate. Small amounts of thickeners also increase the viscosity of the serum.

Lampitt and Bogod(162) detect homogenisation as follows: 50 ml. of milk (of fat content A) are centrifuged in a 100 ml. tube for 2 min. at 200 r.p.m. The fat content (B) of the second 11 ml. withdrawn from the bottom of the tube is determined. If 100 B/A is over 90, the milk is homogenised; if less than 25, there is large admixture of unhomogenised milk; a rough indication of the relative amounts of the two kinds of milk in the mixture can be gauged from the above value.

(*d*) Determination of lactic acid. Citric acid

On boiling cheese with water and barium carbonate, filtering and decomposing the barium lactate with sulphuric acid, the lactic acid can be calculated from the amount of barium sulphate obtained; the method gives slightly high results(163).

Hartmann and Hillig(164) have prepared a serum from the milk product by using a mixture of oxalic acid, acetic acid and potassium acetate. The serum is treated with lead acetate, filtered and the lactic acid liberated by treatment with hydrogen sulphide. Three hours' extraction with ether isolates 98 per cent. of the lactic acid, which is quantitatively oxidised to oxalic acid by alkaline permanganate. After removing manganese with alkaline sulphide, the oxalic acid is separated and deter-

mined as calcium oxalate. The results are slightly high for low, and slightly low for high, lactic acid contents.

The citric acid content of milk averages 0·16 per cent. and, by its determination, the total milk solid content of a food product can be roughly estimated. Milk solids in bread can be determined by this method(165).

(*e*) The solubility of milk powder

Howat and Wright(136) have criticised the accepted methods of determining the solubility of milk powder in that they do not take into account the two types of protein insolubility in dried milk. Wright(166) has suggested the following method. Dried milk (2 g.) and distilled water (20 ml.) are stirred in a boiling tube at 20° C. for 30 min. and then centrifuged at 3000 r.p.m. for 15 min.; the protein of the supernatant liquid is precipitated by trichloracetic acid and this amount, expressed as the percentage of the total protein of the dried milk, gives the solubility. The reconstitution can be carried out at 50° C. to ensure recovery of the solubility of the protein rendered insoluble by dry heat.

Mohr *et al.*(167) use a method embodying the same principles except that glass beads are used to disperse the milk powder in ten times its weight of water. The mixture is centrifuged at 2000 r.p.m. for 10 min., and the dry matter of the supernatant liquid determined. The solubility is then calculated on the dry-weight basis of the original powder.

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